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ABSTRACTS

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Lectures

Biofunctional collagen-like materials: self-assembling collagen-model peptides and peptide-decorated nanoparticles

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Collagen is a protein of considerable biological importance and commercial interest. It is the major structural component of connective tissue including skin, bone, and blood vessels. Also, collagen mediates platelet activation and the formation of hemostatic plugs. The large triple helical domains of collagen consist of three peptide strands of repeating Gly-X-Y triplet motifs, with Pro and Hyp principally occupying positions X and Y, respectively. Our understanding of collagen's structural requirements to achieve biofunctionality can be advanced by the study of peptide subunits known as collagen model peptides (CMPs). We have designed collagen-mimetic peptides based on the concept of hydrophobic self-assembly. Certain 32-mer peptides, $\sim$9 nm in length, can self-assemble into triple helical structures that self-assemble further into micron-size, fibrillar, collagen-like materials. The formation of micrometer-length, composite fibrils was established by DLS, TEM, light microscopy, and AFM. We identified two peptides with robust biofunctionality, which was gauged by their ability to induce platelet aggregation in vitro (i.e., their thrombogenicity). Aromatic rings at the termini were critical for the observed effects, and computational studies supported this point of view. Such self-assembly by aromatic–aromatic interactions offers a straightforward approach to oligomerizing CMPs into biofunctional fibrillar structures. In addition, we explored nanoparticles with CMPs attached onto their surface.

The chemical evolution of amino acids and peptides: the first steps towards the origin of life

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Chemical and information theoretical arguments clearly show that a protein world is the by far most probable starting point of life, rather than RNA or DNA. Simulation experiments have shown that in the geochemical scenario of the primordial earth amino acids can be easily formed also with a neutral or slightly oxidising atmosphere. The combination of the amino acids to peptides and ultimately proteins can be explained by the 'salt-induced peptide formation (SIPF)' reaction. This reaction has numerous properties relevant for contemporary biomolecules and its 'fingerprint' can be identified in the oldest still living organisms. Simulation experiments with this type of peptide formation also supply strong indications, why living organisms almost exclusively use L amino acids in their proteins.

$GABA_A$ receptors: structure, pharmacology, and function

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$GABA_A$ receptors are chloride ion channels that can be opened by GABA. The majority of these receptors are composed of two alpha, two beta and one gamma subunit. Based on the structures of the acetylcholine binding protein and the nicotinic acetyl choline receptor, comparative (homology) models of the combined extracellular and transmembrane domains of $GABA_A$ receptors have been generated. The models indicate that in addition to the already described binding pockets in the extracellular domain there are other water accessible spaces within the $GABA_A$ receptors, which probably allow conformational flexibility of the receptors, but could also serve as drug binding sites. Recently, we established a novel mouse model that allows to rapidly and reversibly enhance or reduce the activity of neurons in the basolateral amygdala and to study the effects of these modulations in animal tests of fear and anxiety. This mouse model is based on the point mutation gamma 2F77I in the gamma 2 subunit of $GABA_A$ receptors, that eliminates the action of the benzodiazepine site agonist zolpidem or the inverse agonist DMCM all over the brain. By a stereotaxic injection of adeno-associated virus vectors expressing the cre-recombinase and the EGFP-labeled wild-type gamma 2F77 subunit, the floxed gamma 2F77I subunit is replaced by the EGFP-gamma 2F77 subunit, and receptors in the infected cells should then be selectively modulated by a systemic application of the above mentioned drugs. This should allow us to investigate the function of neurons in the basolateral amygdala in anxiety and fear related behaviour.

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Development of light-sensitive glutamate receptors

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The ion channels, transporters and metabotropic receptors that underlie neuronal activity can be seen as molecular machines that are amenable to functional manipulation. One of the most rewarding functions to add is sensitivity toward light. Our group has succeeded in photosensitizing glutamate receptors, such as kainate receptors and NMDA receptors, using a combination of synthetic chemistry and protein engineering. In addition, we have developed a "reversibly caged" version of glutamate. I will discuss the principles that underlie the design of artificial photoreceptors and photochromic neurotransmitters and show their applications in cultured neurons, brain slices, and intact animals (e.g. zebrafish, leeches and mice). The usefulness use of these systems in the elucidation of neural circuitry and the restoration of vision will be discussed as well.

The synchrotron (SFTIRM) as an advanced research tool for protein image, protein structure, and protein nutrition research in plant tissues within subcellular dimension

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A synchrotron is a giant particle accelerator that turns electrons into light. It is able to produce extremely bright light, million times brighter than sunlight. The extremely bright synchrotron light makes it possible to detect biomaterial structure (chemical make-up) at molecular and cellular levels. Synchrotron radiation-based Fourier transform infrared microspectroscopy (SRFTIRM) has been developed as a rapid, direct, non-destructive and bioanalytical technique. In contrast to traditional "wet" analytical (chemical) methods which during processing for analysis often result in destruction of the intrinsic structures (e.g. protein) of plant/seed/feed, this cutting-edge synchrotron-based analytical technology, taking advantages of synchrotron light brightness and small effective source size, is capable of exploring the molecular chemistry or structure of a biological tissue without destruction inherent structures at ultra-spatial resolutions. The objective of this research program was to apply the synchrotron-based bioanalytical technique (SRFTIRM) to plant/seed/feed sciences. The results show that with the synchrotron-based bioanalytical technique, the protein intensity and distribution of plant/seed/feed could be imaged within cellular and sub-cellular dimensions, and the plant protein inherent structure could be revealed at ultra-spatial resolutions and related to nutrient availability.

Glutamate receptor immunity and autoimmunity

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Glutamate is the major excitatory neurotransmitter of the nervous system.

Glutamate-receptor immunity: We found that T-cells express very high levels of glutamate/AMPA receptors. This is true for normal, cancer and autoimmune T-cells of human and mouse origin. Furthermore, glutamate by itself ($\sim$10 nM) activates/elevates vital T-cell functions among them: de novo gene expression, adhesion to extracellular-matrix proteins, chemotactic migration, expression of TCR$\alpha\beta$, CD3zeta, CD147 and other important T-cell proteins/receptors, MMP-9 secretion and activity, and T-cell engraftment in vivo. All this suggests glutamate is very important for T-cell function, and that glutamate can be used therapeutically, primarily for augmenting T-cell immunotherapy of cancer. Yet, in T-cell leukemia/lymphoma and T-cell mediated autoimmune diseases, glutamate and/or glutamate receptors should be blocked.

Glutamate-receptor autoimmunity: Autoantibodies to glutamate-receptors are present in some patients and may be neuropathogenic. Indeed, we and others found glutamate/AMPA-receptor-subtype-3 (GluR3) autoantibodies in 35% of patients with different epilepsies, while glutamate/NMDA-receptor-subunits-R2A/B (NR2A or NR2B) autoantibodies (some cross-reacting with dsDNA) were detected in 35% of SLE patients (with or without neuropsychiatric impairments), in 18% of epilepsy patients, and in some patients with stroke and encephalitis.

GluR3-autoantibodies bind neurons and can: activate glutamate/AMPA receptors, kill neurons by either excitotoxicity or complement-activation, cause multiple brain damage, induce neurobehavioral/cognitive impairments, and augment seizure severity in GluR3B-immunized mice. NR2/dsDNA-autoantibodies bind and can subsequently kill hippocampal and cortical neurons. Taken together, we recommend diagnosing and attempting to silence glutamate-receptor autoantibodies in some human neurological diseases, especially in intractable epilepsy and neuropsychiatric lupus, as they may genuinely contribute to the neuropathology.

Amino acid analysis

Amino acid analysis by HPLC after derivatization with 9-fluorenylmethyloxycarbonyl chloride: literature overview, further study and utilization for the analysis of the free amino acids in plasma

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A literature overview is given of HPLC methods currently in use to determine amino acids (AAs) as their 9-fluorenylmethyloxycarbonyl (FMOC) derivatives. On the basis of detailed literature overview an exhaustive derivatization study was performed with 22 AAs, applying photodiode array and fluorescence detection, simultaneously: in order to clear up the controversial points of FMOC derivatization. Model investigations have been carried out as a function of the reaction time and reaction conditions, such as the molar concentration of the reagent, the molar ratios of the reactants, the pH and the solvent composition of the reaction medium. Special emphasis was put (1) on the evaluation of the blank values of the reagents, as a function of the composition and that of the pH of the reaction medium, (2) on the unambiguous quantitation of all AAs, including the less reactive aspartic and glutamic acids, as well as on the formation and transformation of histidine and tyrosine, existing partly, as single (*N*-FMOC-histidine, *N*-FMOC-tyrosine), partly as double labeled species (*N,NH*-FMOC-histidine, *N,O*-FMOC-tyrosine).

Reproducibilities of 22 AAs, including histidine and tyrosine derivatives, obtained under optimum derivatization conditions with acetonitrile containing reagents, at pH 9, derivatization time = 20 min), and characterized with the relative standard deviation percentages of their responses ($\leq$3.98 and 4.35% RSD for standards and plasma samples, respectively). Quantitation limit of AAs varied between 1 and 10 pmole.

D-amino acids as markers for various phenomena in food and biosciences

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Use of advanced chiral resolution techniques (HPLC, GC–MS, LC–MS, CE) developed in the last few decades revealed that a multitude

of D-amino acids (D-AAs) are fairly common in the biosphere, body fluids and tissues, and in the diet. Common bacteria possess a wealth of more or less specific AA racemases and contain both peptidoglycan-bonded as well as free D-AA. Consequently, D-AAs can serve as biomarkers in particular for bacteria which can be also harmful or cause food spoilage. However, many microorganisms are beneficial and are employed for the controlled fermentation of fermented foods such as yoghurt, ripened cheeses, vinegar, wine or soy sauce. Notably, D-AAs are also common in foodstuffs containing glucose or fructose together with amino acids. Examples are concentrated plant saps and fruit juices, dried fruits and vegetables, and honey. Heating experiments of synthetic AMADORI compounds demonstrate that D-AAs are formed at the beginning of the MAILLARD reaction that is defined as interaction between reducing sugars and amino compounds. Another reaction, named α–β transpeptidation of aspartate residues, takes place inevitably in long-living proteins such as teeth dentin, eye lens proteins, and some brain proteins. The intermediately formed aspartyl succinimide is subject of tautomerism leading to racemization of the aspartyl residues. This process, together with subsequent ring opening, results in irreversible conformational changes and loss of biofunctionality of the corresponding proteins.

Amino acid identification and quantification without derivatization applying different detection methods

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The determination of native amino acids is maybe one of the hardest analytical challenges because this class of molecules is constituted of non volatile very small polar molecules. Consequently, they are for long transformed into volatile derivatives to be analysed by gas chromatography. Furthermore, most of them are lacking of a chromophore group. Thus, they are also analysed by liquid chromatography (LC) using a pre or post column derivatization step with a chromophore or fluorophore reagent.

In the last decade, the improvement of instrumentation, the development of new stationary phases and the development of original separation methods relaunch the interest in native amino acid determination. Thus, underivatized amino acids can be nowadays quantified by LC using an evaporative light scattering detector, a chemiluminescence nitrogen detector or a conductivity detector. Structural detectors such as nuclear magnetic resonance and mass spectrometry (MS) were also evaluated. LC coupled tandem MS (MS/MS) allowed rapid, sensitive and specific determinations of native amino acids in very complex samples. Thus, underivatized amino acids were successfully assayed by LC–MS/MS in biological fluids and tissues, etc. The same analytical procedures were extended to the analysis of di- and tripeptides in Champagne wines. LC–MS/MS is still evaluated and is always so promising that the literature is regularly increasing in this field.

This presentation will give a current overview of the quantification of native amino acids involving different separative methods (including capillary electrophoresis) and the above mentioned detectors. The presentation will be illustrated by several applications concerning food or biological samples.

Bioactive fungal peptides containing α-dialkyl α-amino acids

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Among the peptidic metabolites of filamentous fungi, a group named peptaibiotics attracts continuous attention. Peptaibiotics are *N*-acylated peptides which contain non-protein Aib (α-aminoisobutyric acid) and possess antibiotic as well as other bioactivities. Furthermore, peptaibiotics contain protein amino acids and residues such as pipecolic acid, 3-methylproline, 1-aminocyclopropanecarboxylic acid, and C-terminal bonded 2-amino alcohols or polyamines. For the entirety of peptaibiotics produced by a fungus the term peptaibiome is used and the analytical methods employed are named peptaibiomics. Typically, the lipophilic peptides are extracted with organic solvents from the mycelia of fungi growing on agar plates, then extracts are purified by conditioned SepPak C18 cartridges and eluted with methanol. Sequences are determined by HPLC-ESI-CID-MS/MS or related techniques. The presence of Aib is recognized by fragment ions differing by 85.1 Dalton. Cosmopolitan fungi (micromycetes) producing peptaibiotics comprise genera such as *Acremonium*, *Clonostachys*, *Culicinomyces*, *Emericellopsis*, *Hypocrea* with anamorps in *Trichoderma* and *Gliocladium*, *Geotrichum*, *Lecythophora*, *Monocillium*, *Mycogone*, *Niesslia*, *Paecilomyces*, *Stilbella*, *Tolypocladium*, and *Wardomyces* (see monograph cited above). Recently, new bioactivities of peptaibiotics were reported such as neuroleptic properties and inhibition of anti-HIV integrase 1 and amyloid β-peptide formation. Consequently, the peptaibiomic approach is expected to enable the discovery of a vast number of new fungal peptides which, hopefully, display bioactivities for the benefit of mankind.

Amino acid racemization in the course of the MAILLARD reaction

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The reaction of reducing sugars and amino acids (AA) is known as the MAILLARD reaction (MR). Compounds formed from glucose (Glc) or fructose (Fru) and AA at the early stages of the MR are named AMADORI and HEYNS compounds, respectively. We show that heating of D-Glc and D-Fru with various L-AA leads to the formation of the corresponding D-AA, a process called racemization. Heating of reducing sugars (278 mM) and 12 representative L-AA (2.5 mM) in acetic acid (1 M) at 100°C for 24–96 h lead to the fast formation of free D-AA. After 48 h heating with Glc/Fru, relative quantities of D-AA were determined by GC on Chirasil-Val. D-AA detected were: D-Ala (6.5/8.7%), D-Pro (6.6/10.2%), D-Ser (21.2/21.9%), D-Asp (29.3/30.3%), D-Met (8.6/11.7%), D-Phe (8.1/11.7%) D-Phe (8.1/8.9%), D-Glu (13.8/19.3%), D-Tyr (8.8/8.7%), D-Lys (3.9/5.8%). Quantities of D-AA increased on heating for 96 h and exceeded those resulting from the control experiments in which sugars were omitted. In further experiments L-Ala was heated at 130°C/48 h under otherwise almost identical conditions with saccharides and aldehydes and the quantities of D-Ala formed determined (%D in parenthesis): Glc (10.4%), Fru (39.5%), galactose (18.9%), xylose (34.3%), ribose (40.8%), DL-glycerol aldehyde (30.0%), methyl glyoxal (31.6%),

pyridoxal (41.8%; 3 h). Furthermore, heating of synthetic AMADORI compounds such as fructose-L-Phe and fructose-L-Ala provided 30% D-Phe (50 min/200°C) and 15.8% D-Ala (3 h/130°C), respectively.

Amino acid neurotransporters in health and disease: molecular regulation and signalling

Regulation of amino acid neurotransporters for glutamate (GLT1) and glycine (GLYT1) by ubiquitin

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Activation of NMDA receptors requires the simultaneous binding of two neurotransmitters: glutamate and glycine. The synaptic function of these neurotransmitters in glutamatergic synapses is terminated by the action of several glutamate transporters, GLT1 in particular, and one of glycine, GLYT1. To a large extent, the levels of transporters in the various synaptic elements (presynaptic, postsynaptic and glial) are regulated by intracellular trafficking processes that in turn are controlled by various intracellular effectors (interacting proteins and signaling pathways). Here we report that GLT1 and GLYT1 traffic is regulated by ubiquitination. Both the constitutive turnover in the plasma membrane and the accelerated endocytosis of these transporters promoted by the activation of PKC requires the ubiquitination of lysine residues located in their C-terminal intracellular tails. Endocytosis is impaired in the ubiquitin-deficient cell line ts20. Site-directed mutagenesis revealed that for GLT1 the modified lysines are located in a cluster, being Lys477 and Lys526 especially relevant, although Lys550 and 570 might also participate. For GLYT1 (isoform b) lys619 seems to play a prominent role in its endocytosis. Our data suggest that ubiquitination is required for the interaction of these two transporters with the clathrin-dependent endocytic machinery, and suggest that the traffic of these two proteins might be regulated in a coordinated way. It remains to be determined whether recycling of these neurotransporters might be affected in pathologies involving alterations to the ubiquitin system, thereby interfering with its influence excitatory neurotransmission.

Glutamate transporter signalling: molecular and cellular mechanisms

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In 1998, we predicted that glutamate transporter proteins, besides their carrier function, might exhibit receptor-like signaling activities. Our data indicate that one of the direct modes of transporter signaling is triggered by cell death-associated proteases, caspases. Sublethal doses of some of pro-apoptosis agents activate caspases, that cleave glutamate transporters at their cytoplasmic C- or N-terminal domains. Such a proteolytic modification of EAAC1, a first reported case among neurotransmitter carriers, depends on the pattern of phosphorylation of its C-terminus. The truncation of EAAC1 alters cell surface targeting of this transporter, as well as triggers downstream signaling. Such an unusual signaling via glutamate transporters is mediated both by functionally modified, truncated C-terminal domains and by bioactive peptides that are produced by the caspase-dependent cleavage. These soluble peptides contain several short conserved motifs that functionally interact with other cytoplasmic, mitochondrial or nuclear signaling complexes. The truncation of EAAC1 produces peptides that contain a stretch of 8 aa residues present in 82-FIP, a novel RNA-binding protein that also interacts with FMRP, fragile X-mental retardation protein. Soluble C-terminal fragments of EAAT4 could also interfere with protein translation machinery via phosphorylation of PHAS1. C-terminus of GLAST is a component of cytoplasmic scaffolding complex interacting with FXYD2/γ subunit of Na^+, K^+ ATPase. Glutamate-induced activation of GLAST recruits to the cell surface more transporter molecules that are complexed with γ subunit. Thus, glutamate transporters, that represent novel targets for caspases, can act as scaffolding / shuttle proteins and are capable of exhibiting receptor-like, signal-transducing properties.

Inducible expression of GLT1 decreases proliferation of glioma cells and delays progression of tumour in rat

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Gliomas are known to release excitotoxic concentrations of glutamate, a process thought to contribute to their malignant phenotype through enhanced autocrine stimulation of their proliferation and destruction of the surrounding nervous tissue. A model of C6 glioma cells in which expression of the high affinity glutamate transporter GLT-1 can be manipulated both in vivo and in vitro was used in order to investigate the consequences of increasing glutamate clearance on tumour progression. Non-induced cells show modest glutamate uptake and in the presence of L-cystine, these cells tend to release substantial amounts of glutamate. Overnight exposure to doxycycline increased D-[^{3}H]-aspartate uptake, reaching similar capacity as observed in cultured astrocytes. Efficient clearance of exogenously applied glutamate was evidenced in these cells, even in the presence of L-cystine. These cells were grafted in the striatum of Wistar rats and doxycycline was administered after validation of tumour development by magnetic resonance imaging. Both GLT-1 expression examined by immunohistochemistry and glutamate transport activity measured on synaptosomes appeared robustly increased in samples from doxycycline-treated animals. Moreover, these rats showed extended survival times as compared to vehicle-treated animals, an effect that was consistent with volumetric data revealing delayed tumour growth. As constitutive deficiency in glutamate clearance at the vicinity of brain tumours is well established, these data illustrate the potential benefit that could be obtained by enhancing glutamate transport by glioma cells in order to reduce their invasive behaviour.

Excitatory amino acid transporters and the excitotoxic path to motor neuron degeneration in amyotrophic lateral sclerosis

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During the course of human amyotrophic lateral sclerosis and in transgenic mutant SOD1 mice model of the disease, expression and

activity of the glutamate transporter EAAT2 is remarkably reduced. We showed that mutant SOD1 proteins exposed to oxidative stress inhibit EAAT2 by triggering caspase-3 mediated cleavage at a single defined locus, giving rise to two fragments: truncated EAAT2 (trEAAT2) and C-terminus of EAAT2 (CTE). Analysis of spinal cord homogenates from G93A-SOD1 ALS mice reveals CTE to be of higher molecular weight then expected because it is conjugated with SUMO-1. Furthermore, ICC and IP experiments with primary culture of astrocytes and tissue samples from G93-SOD1 mice showed that CTE-SUMO-1 accumulates in promyelocytic leukemia nuclear bodies (PML-NBs). We have incorporated CTE-SUMO-1 gene under the glial specific promoter element (Gfa2) into adenoviral vector (AdV). Confocal analysis confirmed that the cellular expression of AdV-CTE-SUMO-1 was localized in astrocytes PML-nuclear bodies. In spinal cord astrocytes transduced with AdV-CTE-SUMO-1 co-cultured with primary motor neurons we observed that the expression of CTE-SUMO-1 leads to motor neuron toxicity and caspase-3 activation. CTE-SUMO-1 expressing astrocytes mediated motor neuron toxicity after 48 h in co-culture whereas in control CTE or GFP expressing astrocytes there was no sign of toxicity. Toxicity index of motor neurons was assessed by directly counting motor neurons displaying retracted axons (approx. $\geq$3 cell bodies in length). In summary, we have shown that caspase-3 cleavage of the astroglial glutamate transporter EAAT2 could play a role in the degeneration of motor neurons in ALS.

Amino acid nutrition and fetal growth

Impacts of functional amino acids on fetal growth

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Recent studies from animal models (e.g., pigs and sheep) indicate that arginine, branched-chain amino acids (BCAA), glutamine, and proline play important regulatory roles in implantation, as well as embryonic, placental, and fetal development. After the placenta is formed, arginine, ornithine, citrulline and glutamine are unusually abundant in porcine allantoic fluid (e.g., 4–6 mM arginine on day 40) and ovine allantoic fluid (e.g., 10 mM citrulline and 25 mM glutamine on day 60) during early to mid-gestation. These four amino acids represent 70% of total α-amino acid nitrogen in allantoic fluid in early pregnancy. The marked increases (up to 80-fold) in their concentrations in fetal fluids occur during the most rapid period of placental growth, suggesting that they play a critical role in embryonic and fetal development. For example, BCAA are degraded in placentae to form glutamine, whereas proline is a major amino acid for placental synthesis of polyamines. Additionally, arginine and leucine activate the mTOR cell signaling pathway to enhance protein synthesis and cell proliferation. Importantly, dietary supplementation with L-arginine-HCl to gilts fed a conventional diet between day 30 of gestation and parturition increased the number and total litter weight of live-born piglets by 22 and 24%, respectively. Also, intravenous administration of L-arginine-HCl enhanced embryonic survival and fetal growth in ovine models of both experimentally-induced and naturally-occurring intrauterine growth retardation. Thus, through the synthesis of nitric oxide, polyamines, and other metabolites, amino acid nutrition greatly impacts embryonic/fetal survival, growth and development.

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Mechanisms for attenuated insulin secretion in fetal sheep pancreatic islets with placental insufficiency-induced intrauterine growth restriction

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Placental insufficiency causes fetal nutrient deprivation and leads to intrauterine growth restriction (IUGR). The endocrine pancreas plays an important role in fetal life by secreting insulin, an anabolic hormone for which secretion corresponds to nutrient availability, thus playing a role in coordinating fetal growth to nutrient supply. The goal of our research is to understand developmental adaptations in the endocrine pancreas in response to global nutrient insufficiency, and determine how they manifest into adulthood metabolic diseases, such as type 2 diabetes. We used the fetal sheep and induced placental insufficiency and IUGR by exposing the pregnant ewe to elevated ambient temperatures during mid-gestation, resulting in fetal hypoxia and hypoglycemia. At 0.9 of gestation, we found that glucose stimulated insulin secretion (GSIS) was 82% lower in IUGR fetuses compared to controls. This impairment was due to a 78% reduction in β-cell mass and less insulin per β-cell. We did, however, find that the β-cells from IUGR fetuses were able to secrete more insulin relative to total insulin content, indicating stimulus-secretion coupling is improved. In contrast, a fetal sheep model with only hypoglycemia, produced by a chronic maternal insulin infusion, exhibited 45% lower GSIS response and blunted insulin secretion from isolated islets. Together, these data indicate that hypoxia can play an important role in fetal islet outcomes. One mechanism that we have explored for hypoxia is elevated catecholamine concentrations, which negatively correlated with blood oxygen content to inhibit insulin secretion, but IUGR islets appear to become desensitized to chronic catecholamine exposure.

Arginine nutrition and fetal brown fat development

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Neonatal survival in mammals depends on the offspring's ability to maintain core body temperature upon entry into its extra-uterine environment. Metabolism of brown adipose tissue (BAT) for non-shivering thermogenesis generates approximately 50% of the heat in

newborn lambs. In the present study, BAT tissue development was assessed following maternal intravenous administration of arginine-HCl (27 mg/kg bodyweight) thrice daily from days 100–125 of gestation in both undernourished and overnourished singleton-bearing ewes. On day 125, the fetus was removed, weighed, and dissected for fetal tissues. Infusion of arginine increased ($P < 0.01$) its concentrations in both maternal and fetal plasma. Fetal weight was not different ($P > 0.05$) between ewes receiving arginine and saline-infused ewes within nutritional regimen. Fetuses from both undernourished and overnourished arginine-treated ewes had 62% more ($P < 0.01$) BAT tissue/kg fetal weight than fetuses from respective control ewes. Total lipid within BAT was greater ($P < 0.01$) in overnourished than undernourished ewes, but was not affected ($P > 0.05$) by arginine administration. Concentrations of total protein and mitochondrial protein in BAT were not affected ($P > 0.05$) by diet or arginine administration. Western blot analysis showed no difference in VDAC1 (a mitochondrial pore protein) levels between treatments. The mRNA levels for *4E-BP1, NOS3*, *NRF1, NRF2, ODC,* and *RPS6KA1* were similar ($P > 0.05$) among treatment groups. An increase in BAT mass despite no change in concentrations of uncoupling proteins is expected to enhance heat production. Results indicate that maternal arginine administration increases fetal BAT mass which may improve neonatal thermogenic capacity.

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Leucine-rich diet improves fetal muscle protein metabolism from tumor-bearing dams

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The fetal development in conjunction with tumour growth is not a common condition, but when it happens always imposes a delicate situation for both the maternal host and to the foetus. Nutritional supplementation with BCAA's has shown encouraging results on improving the quality of pregnant-tumor-bearing rat. This study examined the muscle protein metabolism of fetal from tumour-bearing dams (W) fed leucine-rich diet (L) compared to control pregnant rats, analyzing the protein synthesis and degradation and the enzymes activities related to protein degradation pathways. The results showed that fetal protein synthesis reduced around 27% in W group, compared to WL and C group. The protein degradation increased 22% in W foetus. The WL group showed protein degradation similar to C. The proteolytic calcium-dependent pathway showed the calpain activity similar in all experimental groups, suggesting that this system has less participation on the fetal muscle catabolism. The activity of lysosomal enzyme cathepsin H was significantly higher in W fetuses. The muscle chymotrypsin-like activity (the catalytic core of proteasome system of ubiquitin-proteasome pathway) increased in tumour groups, although the WL group showed 32% lower activity compared to W group, clearly showing the leucine modulatory effect. The proteosomic subunit 20 s showed low expression in the fetal muscle from tumor-bearing dams. Finally the insulin levels was similar between W and C groups, but was reduced in the groups supplemented with leucine (L and WL). These results stressed indicate that the use of leucine-rich diet is beneficial and improve fetal development under tumour condition.

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Amino acid sensing mechanisms

Phosphoserine sensing in the central nervous system

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L-phosphoserine, also known as L-serine-*O*-phosphate (L-SOP), is the immediate precursor to L-serine in the serine synthesis pathway and is also an agonist at the group III metabotropic glutamate receptors. L-SOP is produced by the enzyme phosphoserine aminotransferase (PSAT) and metabolized to L-serine by phosphoserine phosphatase (PSP). Using a novel analytical procedure, we show that L-SOP is present in rat brain at about the same concentration (5 μM) as dopamine and that it is substantially more potent than L-glutamate at the mGluR4 receptor subtype. Activation of presynaptic mGluR4 inhibits the release of glutamate and GABA.

Immunocytochemical analyses showed that in the rat hippocampus, cells within the subgranular zone were co-labeled with anti-PSP and anti-PSA-NCAM, a marker for neurogenic cells. In the cerebellar cortex, Purkinje neurons expressed relatively high levels of both enzymes while robust expression of PSAT was also observed in the Bergmann glia. L-SOP released from Purkinje neurons or Bergmann glia could activate mGluR4 present on parallel fiber terminals. The presence of L-SOP in brain, its high potency at mGluR4, together with the restricted distributions of the synthetic and metabolic enzymes, suggest that L-SOP might act as an endogenous ligand at Group III metabotropic glutamate receptors in the CNS.

Characterization of GPRC6A: a novel 7TM receptor activated by L-α-amino acids and positively modulated by divalent cations

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We recently reported the cloning and analysis of expression of a novel human family C G-protein coupled receptor, termed GPRC6A. Homology modeling of the GPRC6A ATD based on the crystal structure of the metabotropic glutamate receptor subtype 1 predicted interaction with α-amino acids, and was employed to rationally select potential ligands. Measurement of Ca^{2+}-dependent chloride currents in *Xenopus laevis* oocytes facilitated the deorphanization of GPRC6A and identification of L-α-amino acids as agonists. The most active agonists were basic L-α-amino acids, L-Arg, L-Lys and L-ornithine, suggesting that these may function as endogenous signaling molecules. Mutation of two specific residues of the GPRC6A ligand binding pocket completely abolishes activity of the receptor, supporting the proposed model of GPRC6A. Cloning, cell surface expression and deorphanization of the mouse and rat orthologues further reinforces the assignment of the agonist preferences of human GPRC6A. Most recently, we have developed efficient assays based on measurement of intracellular calcium and inositol phosphate levels in mammalian cells, which have been used to characterize a range of commercially available L-Arg and L-Lys analogs and allosteric

modulation by divalent cations. However, no selective and potent agonist or antagonist has yet been identified and we have therefore developed a GPRC6A knock-out mouse to study the physiological function of the receptor. Initial studies of the knock-out mouse have shown that it grows normally, does not show an obvious behavioral phenotype and does not have a bone phenotype under normal physiological conditions.

L-amino acid sensing by calcium-sensing receptors: agonists and modulators

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Members of a sub-group of class III G-protein coupled receptors are now recognized as having broad-spectrum amino acid-sensing properties and couple to multiple intracellular signaling pathways including those for the regulation of hormone secretion, proliferation and cell fate. The calcium-sensing receptor, like other members of class III, binds amino acids in its homo-dimeric VFT domains and responds sensitively to members of the aromatic, aliphatic and polar sub-classes. Amino acids are positive allosteric modulators of pathways linked to intracellular Ca^{2+} mobilization in CaSR-expressing HEK293 cells and parathyroid cells but have only limited fine-tuning effects on the activation of PI-PLC and ERK1/2. Recent work demonstrates that although amino acids are modulators of several extracellular Ca^{2+}-regulated pathways, they act atypically as agonists of a pathway linked to the control of cAMP accumulation. These results demonstrate that class III broad-spectrum amino acid sensing receptors exhibit highly flexible ligand- and pathway-specific signaling responses.

Aminoprivic sensing: intracellular signaling in the anterior piriform cortex

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The brain's anterior piriform cortex (APC) houses the sensor for indispensable amino acid (IAA) depletion in omnivorous mammals and birds. The glutamatergic output neurons of the APC project to the feeding circuitry in the brain. Behaviorally, an IAA deficient diet is promptly rejected, allowing adaptive foraging and dietary selection. In the sensory mechanism for detection of IAA depletion in the APC, a decrease in an IAA leads to deacylated tRNA and phosphorylation of eukaryotic initiation factor 2 at its alpha subunit (eIF2α) via the general amino acid control kinase, GCN2. Increased *p*-eIF2α serves as an indicator of sensing IAA-deficiency in the primary neurons of the APC. Using electrophysiology, excitatory postsynaptic potential (EPSP) field recordings from APC slices also show neuronal activation (increased amplitude) in response to IAA depletion. Both blockade of glutamatergic AMPA receptors, and removal of extracellular calcium, abolish the postsynaptic potential response with no effect on *p*-eIF2α. Intracellular chelation of calcium with BAPTA-AM or blockade of calcium release via ryanodine receptors each reduced the levels of *p*-eIF2α in APC slices incubated in a threonine devoid (ThrDev) medium and blocked the elevated electrophysiological recording (EPSP) due to ThrDev in the APC slice. These results indicate that intracellular calcium release is required for full expression of both the biochemical and subsequent electrophysiological responses to IAA depletion in the primary sensory neurons of the APC.

Sensing and signaling functions associated with the SNAT2 amino acid transporter

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The SNAT2 ("System A") amino acid (AA) transporter is widely-distributed in mammalian cells mediating the uptake of neutral AAs for protein synthesis and other metabolic processes. A major feature of System A is its upregulation in response to AA limitation, a process referred to as adaptive regulation, which not only involves an increase in SNAT2 gene expression, but an increase in SNAT2 protein stability. SNAT2 adaptation can be suppressed by resupply of any single System A substrate, including Me-AIB, in an otherwise AA deficient environment. Additionally, studies involving shRNA knockdown, expression of transporter chimeras and analysis of cell signalling demonstrate that SNAT2 provides a repressive signal on the one hand for regulation of its own gene during AA sufficiency, whilst simultaneously promoting activation of the mTOR pathway on the other, which stimulates protein synthesis and cell growth.

The concept of a dual function nutrient transporter–receptor (termed a "transceptor") is well established in lower eukaryotes such as yeast, but is only now being recognised to be of importance in the context of mammalian cells. Our findings raise the possibility that SNAT2 acts as a "transceptor" capable of "sensing" AA substrate availability and passing on signals that regulate gene expression and a key nutrient signaling pathway. SNAT2 has thus revealed itself as a potential drug target by which to promote cell growth (e.g. to counteract age-related muscle wasting) or, if an inhibitor is developed, to reduce cell growth (e.g. in cancer chemotherapy).

Aberrant amino acid signalling and metabolism in the Deletor mouse

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We performed transcriptomic, metabolomic and lipidomic profiling in the Deletor mouse, a disease model for a late-onset mitochondrial myopathy and neurodegeneration. These mice exhibit all the biochemical hallmarks of a late-onset myopathy including mitochondrial DNA deletions and COX deficient fibres in skeletal muscle. We have previously shown that the Deletor muscle shows features of starvation response. Here we found amino acid metabolism and related signalling to be dysfunctional in multiple tissues. Transcriptomic analysis of Deletor skeletal muscle revealed that genes involved in amino acid metabolism were induced. We also performed promoter-based bioinformatic analyses of the Deletor skeletal muscle transcriptomic data and found that many of the induced genes contained the same elements induced by a known stress response. Some of the genes induced have not previously been described to be activated by this stress pathway, however, they too contained the stress response elements. We propose that dysfunctional systemic amino acid metabolism and signalling occurs secondarily to respiratory chain dysfunction. Furthermore we also

propose a mechanism by which respiratory chain dysfunction in muscle induces a stress response.

Amino acids, peptides, proteins, exercise and diabetes

Pancreatic beta cell amino acid metabolism in the regulation of insulin secretion

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Individual amino acids do not evoke insulin-secretory responses in vitro when added at physiological concentrations, rather, combinations of physiological concentrations of amino acids or high concentrations of individual amino acids are much more effective. In vivo, amino acids derived from dietary proteins and those released from intestinal epithelial cells, in combination with glucose, stimulate insulin secretion, thereby leading to protein synthesis and amino acid transport in target tissues such as skeletal muscle. Mitochondrial metabolism is crucial for the coupling of glucose, alanine, leucine, glutamine and glutamate recognition to exocytosis of insulin granules. Mitochondria generate ATP (the main coupling messenger in insulin secretion which causes closure of K^+_{ATP} channels, plasma membrane depolarization and Ca^{2+} influx) and other factors, which serve as sensors for the control of the exocytotic process. The main factors that mediate the key amplifying pathway over the Ca^{2+} signal in nutrient-stimulated insulin secretion are nucleotides (ATP, GTP, cAMP, NADPH), although metabolites have also been proposed, such as long-chain acyl-CoA derivatives and glutamate. Aspartate and glutamate are substrates required for the operation of the redox malate-aspartate shuttle, which in the beta cell ensures efficient transfer of glycolytic NADH to the mitochondrial matrix for oxidation. Glutamate may also participate in the formation of glutathione, via reactions of the gamma-glutamyl cycle, ensuring efficient responses to oxidative stress. In addition, after chronic exposure, specific amino acids may influence gene expression in the β-cell which impact on insulin secretion and cellular integrity. Therefore amino acids may play a direct or indirect role in insulin secretion.

Structural studies of glucagon family peptides

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Incretin peptide hormones GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) play an important role in postprandial nutrient homeostasis in man. These hormones have an important role to play in metabolism since incretins have multiple glucose lowering abilities including promotion of insulin biosynthesis and secretion in the pancreatic beta-cell. There has been a keen interest in the potential of stable forms of incretin hormones of GLP-1 and GIP in diabetes therapy.

We have shown that GIP(1-30)amide produced a similar dose-dependent insulinotropic pattern to native GIP. NMR structural studies of GIP carried out in our laboratory under physiological conditions showed that the GIP forms an alpha-helical conformation between residues Ser11-Gln29 and its bioactive conformation and hydrophobic character. Current NMR studies of GIP in phospholipid media show that the GIP adopt an alpha-helical conformation between residues 11–30 but C-terminal remains to be random coil. Further studies were conducted to advance our understanding of the likely interactions of the native intact GIP(1-42) structure with the extracellular domain its GPCR receptor. This work using NMR in a micelle background examined the likely docking position of GIP with its receptor binding region and revealed a likely interaction of various GIP amino acid side chains with specific residues on the extracellular domain from the human GIP receptor.

Metabolic responses of obestatin peptides in normal and high-fat fed mice

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Obestatin is a peptide derived from the preproghrelin gene that has been demonstrated to alter food intake, gut motility and body weight. This investigation assessed acute metabolic responses to feeding following administration of obestatin (1–23) or truncated peptides (11–23) or (1-10) to normal and high-fat fed (HFF) mice. Before assessing effects on feeding, direct effects of obestatin peptides (1 μmole/kg; $n = 6$) on glucose regulation or insulin secretion were examined in normal mice following glucose challenge (18 mmol/kg). No alterations in plasma glucose or insulin responses were observed. Following fasting for a 16 h period, normal or HFF mice received either intraperitoneal obestatin (1–23), (11–23) or (1–10) (1 μmole/kg; $n = 6$) 4 h prior to an allowed 15 min period of feeding. In both normal and HFF mice obestatin (1–10) did not affect feeding or subsequent metabolic responses. Obestatin (1–23) and (11–23) reduced food intake in normal mice by 43 and 53% ($P < 0.05$), respectively, however, reductions in food intake in HFF mice were greater (86 and 90% lower than saline, respectively; $P < 0.01$–0.001). Furthermore, metabolic responses to feeding differed in normal mice compared with HFF mice. In normal mice both glucose excursions and insulin responses were lowered by 64–77% ($P < 0.01$–0.001) compared with saline controls. In HFF mice glucose responses to feeding after obestatin (1–23) and (11–23) administration were reduced by 58 and 55%, respectively. However, in contrast to normal mice the plasma insulin responses of HFF mice remained unchanged following injection of obestatin peptides. These observations support a role for obestatin in regulating metabolism through changes of appetite, but do not indicate direct actions on glucose homeostasis or insulin secretion. Furthermore, the inhibitory effects of obestatin (1–23) and (11–23) on feeding appear to be greater in HFF mice than normal mice and the insulin responsiveness to feeding is impaired.

Aminoacyl-tRNA synthetases in signaling and diseases

Overall proteome quality sensing and maintenance is governed by the ER

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The gene translation machinery is directly linked to a large number of human diseases. Our laboratory is focused on studying the reactions of tissues and cells to errors caused by aminoacyl-tRNA synthetases (ARS), and tRNAs. We study the connections between ARS biology and human disease in a number of interlinked projects that aim at increasing our understanding of these connections as well as generating new tools for the treatment of the associated diseases.

A decrease in the fidelity levels of the protein synthesis apparatus has been shown to be deleterious and potentially lethal. Several studies in bacteria, yeast, and human cells have demonstrated that an increase in the rate of error during protein synthesis reduces cell viability. In mammals, the malfunctioning of some elements of the translation machinery causes disease. We have modified a specific ARS-tRNA interaction to increase the frequency of proteome beyond 10^{-4} in a controlled manner, and to study the response that this problem elicits in human cells and in a chicken embryo model. The impact of these substitutions is being characterized, quantified and modeled. We have been able to show that cells monitor proteome quality by surveying only a subset of their proteins.

Role of aminoacyl-tRNA synthetases in the peripheral nervous system: lessons from *Drosophila*

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Aminoacyl-tRNA synthetases (AARS)—the enzymes charging tRNA with its cognate amino acid- are key components of the protein synthesis machinery. Whilst their activity is clearly required by all cells, it is unexpected that defects in several AARS have been recently reported to have highly specific consequences in the nervous tissue and cause neurodegenerative disorders.

Drosophila melanogaster is an attractive model organism for studying the AARS role in neuronal homeostasis. Loss of GlyRS, TrpRS or GluRS activity in fly neurons specifically affects the dendritic and axonal arborization, indicating that different aspects of neuronal morphogenesis are differentially sensitive to perturbations in translation.

We have reported that mutations in TyrRS cause dominant intermediate Charcot-Marie-Tooth neuropathy type C (DI-CMTC). This is a novel form of inherited neuropathy caused by axonal degeneration and demyelination of peripheral nerves. Our biochemical and genetic complementation experiments demonstrated that DI-CMTC associated TyrRS mutations differentially affect its aminoacylation activity and therefore the disease is not caused by loss of the canonical enzymatic function. Pursuant to understand the mechanistic link of TyrRS to neurodegeneration, we established a *Drosophila* DI-CMTC model and tested if the disease-causing human mutations could cause neuropathy symptoms in a dominant way in flies. Ubiquitous and neuron-specific expression of mutant TyrRS in *Drosophila* recapitulated several hallmarks of the human disease, indicating that DI-CMTC mutations in this ubiquitously expressed enzyme have intrinsic effects in neurons. Our results show that the connection of TyrRS to neurodegeneration is deeply rooted in evolution and that this connection does not result from haploinsufficiency of the canonical aminoacylation function.

Mechanism of tRNA-dependent incorporation of free radical damaged amino acids into proteins

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The accumulation of proteins damaged by reactive oxygen species (ROS), conventionally regarded as having pathological potentials, is associated with age-related diseases such as Alzheimer's, atherosclerosis, and cataractogenesis. Exposure of the aromatic amino acid (aa) phenylalanine to ROS-generating systems produces multiple isomers of tyrosine—*m*-tyrosine (*m*-Tyr), *o*-tyrosine (*o*-Tyr) and the standard *p*-tyrosine (Tyr). Previously it was demonstrated that exogenously supplied, oxidized aa could be incorporated into bacterial and eukaryotic proteins. It is, therefore, likely that in many cases, in vivo damaged aa are available for de novo synthesis of proteins. Though the involvement of aminoacyl-tRNA synthetases (aaRSs) in this process has been hypothesized, the specific pathway by which ROS-damaged aa are incorporated into proteins remains unclear. We provide herein evidence that mitochondrial and cytoplasmic phenylalanyl-tRNA synthetases (*Hsmt*PheRS and *Hsct*PheRS respectively) catalyze direct attachment of *m*-Tyr to $tRNA^{Phe}$ thereby opening up the way for delivery of the misacylated tRNA to the ribosome and incorporation of ROS-damaged amino acid into eukaryotic proteins. Crystal complexes of mitochondrial and bacterial PheRSs with *m*-Tyr reveal the net of highly specific interactions within the synthetic and editing sites.

Genetic code expansion by non-canonical tRNA synthetases

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The genetic code strictly assigns 64 codon triplets to the 20 canonical amino acids, except for three stop codons, UAG (amber), UGA (opal) and UAA (ochre). These stop codons are occasionally recoded to an amino acid by specialized transfer RNAs (tRNAs) called suppressor tRNAs. In all living cells, UGA opal codon is recoded to selenocystein (Sec), the 21st natural amino acid, depending on the downstream specific RNA element. The incorporation involves a tRNA synthetase and two metabolic enzymes. We elucidated the structural basis of the indirect amino acid conversion to expand the genetic code. In methanogenic archaea and some anaerobic eubacteria, the UAG amber codon is recoded to pyrrolysine, the 22nd amino acid, through direct acylation of amber suppressor $tRNA^{Pyl}$ by pyrrolysyl-tRNA synthetase (PylS). The $tRNA^{Pyl}$ suppressor has an unusual structure, lacking most of the consensus features of canonical tRNAs, analogous to mammalian mitochondrial tRNAs. It is thus unclear how such unusual tRNAs function in the ribosome and how PylS discriminates

between suppressor and canonical tRNAs. Here we solved the crystal structure of the PylS·tRNA^{Pyl} complex from *Desulfitobacterium hafniense* at 3.1 Å resolution. The suppressor tRNA^{Pyl} has an unusual minimal core structure formed by a non-standard D-loop, a TΨC-loop and a variable loop, which are re-organized to mimic the canonical L-shape to function normally in the ribosome. PylS recognizes the unusual compact core structure by steric compatibility, which enables discrimination of the suppressor from the other canonical tRNA.

Human aminoacyl-tRNA synthetases as multiplayers in signaling and diseases

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Aminoacyl-tRNA synthetase (ARSs) are ancient enzymes linking amino acids to their cognate tRNAs. While these enzymes are catalytically involved in protein synthesis, they also play diverse regulatory roles in the determination of cell fate via their versatile molecular interactions and secondary catalytic activity. For instance, several different ARSs are secreted as cytokines with distinct activities in angiogenesis and immune responses. In higher eukaryotic systems including human, nine different ARSs form an intriguing macromolecular protein complex with three non-enzymatic factors named as AIMP1/p43, AIMP2/p38 and AIMP3/p18. These factors are not only important for the cellular stability of the associated ARSs but also implicated in various pathophysiological processes. For this reason, disruption of gene expression or detrimental mutations of ARSs and AIMPs are associated with many human diseases such as cancer and neurodegeneration. AIMP1 covers broad range of cell regulation and metabolism as secreted cytokine or hormone but also as intracellular signal mediators. AIMP2 and AIMP3 are potent tumor suppressor working at immediate upstream of p53 for the integrity of chromosome. The functional significance and unique working mechanisms of ARSs and AIMPs provide an opportunity that they can be explored as novel disease-associated biomarkers and therapeutic targets.

Basic mechanisms of glutamatergic synaptic transmission and plasticity

Regulation of glutamate release during hippocampal synaptic plasticity

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Hippocampal CA1 pyramidal neurons receive two major classes of excitatory glutamatergic inputs. Direct information from cortex is relayed by the perforant path (PP) synapses whereas information processed by the hippocampus is relayed by the Schaffer collateral (SC) synapses. Although a great deal is known about the properties of the SC synapses and the importance of long-term potentiation (LTP) of synaptic transmission at these inputs for learning and memory, relatively little is known about the properties of the PP synapses and the mechanisms of PP LTP. We compared the presynaptic properties of PP versus SC inputs and examined the possible contribution of changes in presynaptic function to the expression of PP LTP.

Presynaptic properties were assayed in acute hippocampal slices from adult mice using two-photon microscopy of a fluorescent marker of synaptic vesicle cycling, FM 1–43. The rate of dye release from presynaptic boutons during synaptic stimulation provided an index of the efficacy of presynaptic function. Under basal conditions, the rate of dye release was two-fold faster at the SC synapses compared to PP synapses, due to a lower contribution of N-type voltage-gated Ca^{2+} channels to the release process at the PP synapses. Induction of PP LTP, which required activation of both NMDA receptors and L-type Ca^{2+} channels, increased the rate of FM 1–43 release due to a recruitment of N-type channels to the release process. Thus, distinct glutamatergic inputs converging on a common postsynaptic cell can differ in their release efficacy, which can be enhanced during long-term potentiation.

Activity-dependent plasticity expressed by NMDA receptors in the hippocampus

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While most excitatory synapses express both AMPA and NMDA subtypes of glutamate receptors, synaptic plasticity (i.e. LTP/LTD) is commonly associated with changes of AMPAR-mediated synaptic transmission. Growing evidence suggests that NMDARs can also express LTP/LTD at CNS synapses. An interesting case can be found at the mossy fiber to CA3 pyramidal cell synapse (mf-CA3) in the hippocampus. Thus far, the focus at these synapses has been on a purely presynaptically induced and expressed form of LTP of basal AMPAR-mediated transmission (classical *mfLTP*). We have recently found that mf-CA3 synapses can also undergo a novel form of plasticity that is expressed postsynaptically as a selective, long-lasting increase in NMDAR-neurotransmission (*NMDAR-mfLTP*). Induction of NMDAR-mfLTP requires coactivation of NMDA and mGlu5 receptors and postsynaptic calcium rise. Unlike classical LTP, expression of NMDAR-mfLTP is due to a PKC-dependent recruitment of NMDARs specifically to the mf-CA3 synapse via a SNARE-dependent process. Recent studies have shown that the synaptic adhesion molecule Neurolgin 1 (NL1) is involved in recruiting NMDARs to synapses. We found that postsynaptic NMDAR-mfLTP, but not classical presynaptic LTP, is substantially reduced in the NL1-KO mice. Moreover, the potentiation of mf-NMDAR-EPCSs induced by loading CA3 pyramidal cells with the catalytically active form of PKC, PKM, is also reduced in NL1-KO mice, strongly suggesting that NL1 is operating downstream of PKC to enhance NMDAR signaling in NMDAR-mfLTP. Our findings at the mf-CA3 synapse strengthen the notion that NMDAR-mediated transmission is more dynamic than originally thought.

Fear learning and synaptic plasticity

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Auditory cued fear conditioning is a widely used associative learning paradigm in which animals learn to associate a neutral stimulus, usually a pure tone, with an aversive stimulus, e.g. a foot shock. Subsequently, animals show fear-related behaviour when the CS is

presented alone. In the amygdala, a putative site of tone-shock association, auditory and sensory input converge onto individual cells and projections from the amygdala to output brain areas, e.g. the brainstem, mediate fear behaviour. We investigated mechanisms of synaptic plasticity at glutamatergic inputs from the auditory thalamus on neurons of the lateral amygdala during learning. We find that recombinant AMPA type receptors carrying an electrophysiological tag are not incorporated into synapses during basal conditions, however, upon fear conditioning. Interfering with the trafficking of endogenous GluR1-containing AMPA receptors strongly impairs memory formation. These findings indicate that learning and memory formation trigger similar processes as they had been described previously in culture during artificial induction of synaptic plasticity.

Coincidence detection at glutamatergic synapses in the amygdala

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During auditory fear conditioning, the conditioned stimulus (CS) converges in the lateral nucleus of the amygdala with the painful unconditioned stimulus (US) arising from the somatosensory cortical and thalamic areas. This convergence results in potentiation of synaptic responses in auditory inputs to the LA, retaining memory of the CS–US pairing via mechanisms of NMDA receptor-dependent long-term potentiation. NMDAR function requires simultaneous activation of its glutamate and glycine binding sites. We studied the mechanisms of modulation of the NMDAR glycine site in the LA utilizing whole-cell recordings in slices. We found that the glycine site of the NMDAR in LA neurons is not saturated by the endogenous coagonist under baseline conditions. The size of the NMDAR mEPSCs was increased in response to the exogenously-applied glycine site agonists, glycine or d-serine. This finding suggests that activity-dependent release of the endogenous co-agonist of the NMDAR might determine the level of NMDAR activation. Consistent with this, we observed activity-dependent enhancements of the NMDAR function at thalamo-amygdala synapses through increased activation of the glycine site by the endogenous glycine site agonist. Incubation of amygdala slices in D-amino acid oxidase (DAAO)-containing solution decreased the size of the NMDAR-mediated component of glutamatergic synaptic events, suggesting that D-serine might serve as an endogenous activator of the NMDAR glycine site in the amygdala. Such synaptic mechanisms could mediate coincidence detection during simultaneous activity of groups of pre- and postsynaptic neurons and, thus, contribute to the induction of pathway-specific synaptic plasticity in fear conditioning circuits.

G-protein coupled receptors signal selectively to NR2A versus NR2B containing NMDA receptors and thereby differentially control bidirectional synaptic plasticity of CA3–CA1 synapses

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The induction of LTP at CA3–CA1 neurons requires a Src-dependent enhancement of NMDA receptors (NMDAR). It is unclear whether or not plasticity of NMDAR contributes to this induction. We report that NMDAR can also undergo a Src-dependent potentiation which contributes to the induction of LTP of AMPA receptor-mediated EPSCs. Therefore, NMDAR activity regulates plasticity at these synapses. NMDAR in CA1 pyramidal neurons are tetramers predominantly composed of NR1A in combination with either NR2A or NR2B subunits. The role of specific subtypes of NMDAR in the induction of synaptic plasticity has proven to be controversial. We have established that each of the NR2AR (2NR2:2NR2A) and NR2BR (2NR1,2NR2B) subtypes is a selective target of a different G-protein coupled receptor signaling pathway. GPCRs which signal via a sequential Gαq/PKC/Pyk2/Src kinase cascade enhance the activity of NR2AR but not NR2BR. In contrast, GPCR-mediated (Gαs) signals via PKA/Fyn enhance NR2BR and not NR2AR. GPCRs that selectively target NR2AR dynamically shift the relationship between the frequency of stimulation and the direction of synaptic plasticity to the left (lower the threshold of induction LTP) without changing the maximal amplitude of LTP. In contrast, GPCRs acting via Gαs enhance the induction of long-term depression and elevate the threshold for LTP induction. Therefore, NMDAR subtypes play a key role in determining the direction of synaptic plasticity at CA3–CA1 synapses.

Clinical proteomics in early and predictive diagnosis

Predictive diagnostics, targeted preventive measures and personalised treatment: new philosophy in health care

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Personalised Medicine is a new philosophy in healthcare: there is an obvious trend in the paradigm-change from curative medicine to predictive diagnostics, development of well-timed prevention and individual therapy-planning. Innovative biotechnologies such as clinical proteomics represent the essential technological platform for the overall progress in favour of predictive and personalised medicine as the medicine of the future. Amongst the most important aspects are well-organised population screening, targeted prevention of the frequent pathologies, non- or minimally-invasive diagnostics, optimal therapy planning, personalised patient treatment, substantial improvement of the quality of life and optimal solutions for particular social, ethical as well as serious economical problems. This decisive progress can be achieved only by the following well-coordinated measures:

- adequate investment creating novel technologies,
- development of non- or minimally-invasive diagnostic tools,
- well-organised process for exchange and transfer of knowledge among biomedical research entities and biotechnological industries for production of advanced diagnostic tools,
- quality assurance through the introduction of international standards for technological tools and devices, patenting and licenses,
- correct professional education in terms of the application of biotechnological high-tech procedures in medicine,
- intelligent political regulations in the healthcare sector including introduction of obligatory guidelines and clear regulations for the health insurance industry to ensure patients needs are met,

- measures to ensure confidentiality of patient information and personal databank,
- distribution of relevant information among healthcare professionals and users.

Mission of the European Coordinator in this field is performed by "European Association for Predictive, Preventive and Personalised Medicine".

Risk factors and potential prediction in glaucoma

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Why early diagnostics is essential for glaucoma treatment?

Worldwide, 67 million patients are affected by the neurodegenerative eye disease Glaucoma. Glaucomatous optic neuropathy is the second leading cause of permanent vision loss. Early diagnostics has been demonstrated to be highly beneficial for well-timed treatment measures to suppress the disease progression.

What is the impact of predictive molecular diagnostics in glaucoma?

Molecular pathomechanisms of glaucoma demonstrate both a considerable overlap and remarkable particularities to some other neurodegenerative disorders, e.g. Alzheimer's and Parkinson's diseases. Therefore, the selection of a set of key molecules, the expression levels of which are specifically affected by glaucoma, is particularly valuable for the development of highly precise molecular diagnostic approaches.

Noninvasive imaging technologies in early and predictive glaucoma diagnostics.

Imaging of DNA-damage and -repair capacity: Comparative "Comet Assay" analysis demonstrates significantly enhanced DNA-damage and decreased DNA-repair capacity in circulating leukocytes of glaucoma patients *versus* these of controls. Furthermore, the pathology-specific comet patterns have been registered.

Altered gene expression patterns in blood: The following key pathways are affected in glaucoma pathology: stress response, apoptosis and DNA-repair, adhesion, blood–brain-barrier-breakdown, tissue remodeling, transcription regulation, multi-drug resistance, energy metabolism. These pathology-specific molecular patterns in blood may create the basis for the development of novel noninvasive molecular imaging technologies in early and predictive glaucoma diagnostics.

Cancer predisposition in *Diabetes Mellitus*: predictive proteome profiling and personalised therapy planning

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Diabetes mellitus (DM) is a lifelong, progressive disease and worldwide the fourth leading cause of death: every 10 s one patient dies from DM-related consequences. The recent years have brought new knowledge concerning the predisposition to cancer in diabetics: the large population studies performed indicate an increased risk of liver, pancreas, bladder, digestive and urinary tracts, and endometrium cancer types in DM with some age-specific differences and gender-dependent preferences. Although the molecular pathomechanisms of secondary diabetic complications require further investigations, recently collected research data contribute well to understanding of the pathology. Oxidative damage to DNA is well documented for diabetics and can be explained by impaired glucose/insulin metabolism. These findings indicate an imbalance between the increased production of ROS and decreased DNA-repair capacity in DM. Moreover, excessive ROS production leads to induced oxidative damage of chromosomal/mitochondrial DNA. Mitochondrial dysfunction is resulting in decreased energy production. Thus, insufficient repair capacity consequently leads to the accumulation of damaged chromosomal/mitochondrial DNA. Whereas long-term accumulation of DNA mutations is well-acknowledged as triggering cancer, the dysfunction of mitochondria might also be implicated in pathomechanisms of diabetes-provoked cancer. The pathology-specific alterations in protein-profiles of cancer-diseased diabetics versus non-diabetic cancer patients and healthy controls have been demonstrated. The pathology specific marker-candidates profiling in blood samples might be potentially useful for the development of non-invasive predictive diagnostic tools which can be applied to improve the diabetic care.

Predictive diagnostics of long-term outcomes in perinatal asphyxia

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Birth asphyxia is the most frequent perinatal complication annually resulting in some million deaths. This pattern diversifies geographically and extends between 1 and 50 cases per 1,000 births. Several severe pathologies occurring in childhood and adulthood may be primarily triggered via birth asphyxia. The discovery of its molecular pathophysiology would be a breakthrough and the expression profiling of the pathology-specific protein patterns involved in its pathomechanism, is showing great potential.

For the profiling of differentially expressed groups of genes, the smart "gene hunting"—technology of subtractive hybridization, has been used to compare the molecular patterns in brain of asphyxiated newborn versus those in normoxic ones. Following pathways were identified to be affected in brain by the asphyxia: several central metabolic pathways (e.g. energy metabolism, metabolism of nucleic acid and lipids), regulation of nuclear and DNA-binding factors, transcription and translation regulation, free-radical production and redox-control, synthesis of signaling molecules (such as nitric-oxide) and heat-shock proteins, membrane transport, ion-handling, activation of proto-oncogenes. Extensive alterations in the above listed pathways have been reported also at the translational level by the pathology-specific protein profiling. These alterations may be implicated in the development of asphyxia-related chronic complications: (neuro)degenerative processes, generation of pre-cancerous lesions in the affected organs, etc. Pathology-specific protein-profiling by blood tests are highly attractive tools to develop non-invasive diagnostic approaches and to predict an individual predisposition to secondary complications in asphyxiated newborns.

Protein analysis, data mining and information systems in routine predictive laboratory diagnostics

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Background: Judging by the chemical structure, in the routine clinical laboratory well over 60% of analytes belong to the proteins, be them enzymes, protein hormones, immunoglobulins, tumor markers, specific proteins and other related structures. The number of the analytes of protein nature continually increases and so does the number of accumulated data stored in the databases of the laboratory information systems. Latest development in the field of proteomics will increase this number even faster.
Conclusion: Routine clinical laboratories should prepare for their key role in the emerging concept of predictive, preventive and personalised medicine since they have direct access to the data which can serve as the basis for the discoveries of new relations and correlations between analytes of different characteristics. The protein networks scientists try to draw and model may get more reliable when "tuned" using real population data and the latest information technology available. Neural network data mining of clinical databases should be engaged in the laboratory information systems to create reliable and continually evolving models of proteins networks and consequently the model of the entire human metabolism. Such models may then serve in the routine practice of the clinical laboratory for the prediction of possible health problems of the individual patient. The more new analytes are taken into consideration for data mining the better and faster we can have reliable models. Research in the field of proteomics and its routine application has very high potential of boosting the field of predictive, preventive and personalised medicine.

Analysis of potentially toxic food contaminants: proteome of *Aspergillus ochraceus*

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Ochratoxin A (OTA) is a potent nephrotoxic fungal secondary metabolite produced by various moulds including *Aspergillus ochraceus*. OTA is widely distributed in Europe and North America and has been found as a natural contaminant in agricultural commodities, apparently causing ochratoxicosis and nephropathy by degradation of the proximal tubules followed by interstitial fibroses. The toxin has also been implicated in human Balkan nephritis. OTA has been shown to be carcinogenic, teratogenic and immunotoxic as well as foetotoxic.

In this paper we report for the first time on the identification and characterization of major proteins of *A. ochraceus*. To obtain a comprehensive overview about the expression of proteins under different physiological conditions a proteomic approach was applied. For this purpose *A. ochraceus* NRRL 5175 was cultured in malt extract broth. Cell disruption was performed by grinding the mycelium with liquid nitrogen. Proteins were extracted using lysis buffer and precipitated with TCA-acetone and then washed several time. The extracted proteins were separated in a 2D-gel electrophoresis system. The spots were extracted and trypsin digest was carried out. Proteins were identified using MALDI-TOF-TOF mass spectrometer. The verification of results was done using standard database search engines (e.g., MASCOT). Due to incomplete proteome database of this specie standard search engines did not provide optimal confidence in protein annotation. The proteins with low confidence score were de novo sequenced and the generated sequences were subjected to homology search for the identification of proteins.

Cognitive enhancement and $GABA_B$-receptors $GABA_B$ receptor: a complex allosteric machine to tune up synaptic transmission

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The G-protein coupled receptor activated by the neurotransmitter GABA is made up of two distinct subunits, $GABA_{B1}$ that binds agonists, and $GABA_{B2}$ that activates G-proteins. Each subunit comprises an extracellular domain and a transmembrane heptahelical domain. How the two subunits communicate in the heteromeric receptor remains unknown and understand these processes will enable to develop better drugs. Here, we used a combination of biophysical, biochemical and bioinformatics approaches to investigate the molecular functioning of the $GABA_B$ receptor. First, by using a glycan wedge scanning approach, we demonstrate that a relative movement the two extracellular domains is required for receptor activation. Second, we set up a novel multi-labeling approach compatible with time-resolved FRET based on the use of ACP- and SNAP-tag technologies to verify the heteromeric assembly of $GABA_B$ receptors made of wild-type and mutated subunits. We show a direct allosteric coupling between the two heptahelical domains is a key step for receptor activation. Our data are challenging the actual view of the activation process of the $GABA_B$ receptor.

The $GABA_B$ receptors and action of antidepressant drugs

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Depression is a psychiatric disorders with high morbidity and comorbidity. Because of multiple adverse effects of existing drugs and their low efficacy, antidepressant therapy for a substantial number of patients is unsatisfactory. It is widely accepted that GABA is the primary inhibitory transmitter substance in mammalian central nervous system. Data are accumulating that the recently cloned $GABA_B$ receptors might become a target for the development of new antidepressant drugs. The experiments demonstrate, that the most consistent finding following

chronic administration of antidepressants is an increase in $GABA_B$ receptor function, with or without a change in receptor binding or subunit expression. Data are accumulating that antagonists (but not agonists) of $GABA_B$ receptor may have antidepressant effects in the preclinical studies. $GABA_B$ receptor antagonists CGP 36742 and CGP 51176 exhibit antidepressant-like activity in the forced swim test (FST) in mice, $GABA_B$ receptor agonist CGP44532 was not effective in this test, however it counteracted the antidepressant-like effects of CGP 51176. Furthermore when a noneffective dose of $GABA_B$ receptor antagonist was administered together with a ineffective dose of antidepressant drug imipramine, an antidepressant effect was demonstrated in the FST. $GABA_B$ antagonists, CGP 36742 and CGP 51176, were effective in an olfactory bulbectomy (OB) model of depression in rats, the latter one was also effective in chronic mild stress (CMS) rat model of depression. The immunohistochemical data show that GABA-ergic neurons are immunoreactive for calcium binding proteins calbindin and parvalbumin. The number of calbindin immunopositive neurons is reduced by 18.6% in stratum granulosum of dentate gyrus and in the medial prefrontal cortex a significant 17% reduction in parvalbumin immunoreactivity and 20% reduction in calbindin immunoreactivity were observed in rats exposed to chronic unpredictable stress, which can be regarded as a model of depression. The data are in line with the hypothesis of GABA-hypofunction in depression.

$GABA_C$ antagonists as memory enhancers

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Cis- and *trans*-(3-aminocyclopentanyl)butylphosphinic acid (*cis*- and *trans*-3-ACPBPA) and (*R*) and (*S*)-(4-aminocyclopentenyl) butylphosphinic acids ((*R*)- and (*S*)-4-ACPBPA are conformationally restricted analogs of the orally active $GABA_{B/C}$ receptor antagonist (3-aminopropyl)-n-butylphosphinic acid (CGP36742 or SGS742). These compounds were developed and evaluated on recombinant human GABA receptors expressed in *Xenopus* oocytes. These compounds had little effect on $GABA_A$ and $GABA_B$ receptors but were competitive antagonists at human $GABA_C$ receptors being >100-times more potent at $GABA_C$ than either $GABA_A$ or $GABA_B$ receptors. These agents were evaluated in rodents on a number of learning and memory paradigms by intraperitoneal administration. It was found that all four compounds enhance learning and improve memory. As the physiological effects of the $GABA_C$ agents are similar to those reported for CGP36742 (SGS742), it is proposed that the memory effects of SGS742 may be due in part to its $GABA_C$ activity.

Memory enhancing effects of some aminophosphinic acid analogs of GABA

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Aim: To examine the nootropic effects of the $GABA_C$ antagonists (*R*)- and (*S*)-(4-aminocyclopentenyl)butylphosphinic acids (ACPBPA).

Methodology: (R)- and (S)-4-ACPBPA were evaluated in 7–9 week old male Swiss mice using the holeboard habituation test, and the novel object recognition test (NORT). For the holeboard habituation, animals (10 per group) were injected *ip* with saline (vehicle), and the drugs (100 mg/kg). The number of nose pokes was recorded for 10 minutes on day 1 and again 24-h later (day 2). For the NORT, animals (10 per group) were injected *ip* with saline (vehicle), 150 mg/kg SGS742, 0.3 mg/kg scopolamine and (R)-4-ACPBPA (0.1–100 mg/kg) 20 min prior to training. Vehicle and 100 mg/kg (R)-4-ACPBPA were also evaluated when administered immediately after training. The reversal of a scopolamine induced memory loss was also examined using the NORT, with animals injected *ip* with 0.3 mg/kg scopolamine alone, +150 mg/kg SGS742, or +100 mg/kg (R)-4-ACPBPA.

Results and discussion: (R)- and (S)-ACPBPA showed a significant ($P < 0.01$) decrease in nose pokes in the holeboard habituation on day 2 compared to vehicle, indicating improved memory of the holeboard apparatus. In the NORT, the recognition index during retention was significantly increased ($P < 0.05$) in animals treated with SGS742 and 3, 10, 30 and 100 mg/kg (R)-4-ACPBPA before training compared to vehicle. SGS742 (150 mg/kg) and (R)-4-ACPBPA (100 mg/kg) significantly ($P < 0.01$) reversed the induced memory loss observed with scopolamine. (R)-4-ACPBPA administered after training significantly ($P < 0.01$) increased the recognition index compared to vehicle, indicating improved memory. These results show that these $GABA_C$ antagonists effectively enhance learning and memory.

Epigenetic-mediated effects on gene expression and behavior

Dynamic chromatin remodeling events in hippocampal neurons are associated with NMDA receptor-mediated activation of *Bdnf* gene promoter 1

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Activation of brain-derived neurotrophic factor (*Bdnf*) transcription involves changes in chromatin structure through changes in DNA methylation and posttranslational histone modification. In particular, NMDA receptor activation plays an essential role in normal brain function and neuronal survival. To determine the epigenetic events associated with a major activation-dependent *Bdnf* promoter, we screened 12 loci across 4.5 kb of genomic DNA 5′ of the transcription start site (TSS) of promoter 1 for specific changes in histone modification and transcription factor binding following NMDA receptor stimulation by hippocampal neurons in culture,

Chromatin immunoprecipitation (ChIP) assays showed a durable, time-dependent decrease in histone H3 at lysine 9 dimethylation (H3K9me2) within three hours of NMDA receptor stimulation across multiple loci. Concomitant increases in H3K4me2 and H3K9/14 acetylation (H3AcK9/14) were associated with transcriptional activation, but occurred at fewer sites within the promoter. The decrease in H3K9me2 was associated with release of HDAC1, MBD1, MeCP2, and REST from specific locations within promoter 1, although with different kinetics. In addition, occupancy of sites proximal to and distal to the TSS by the transcription factors NF-κB, CBP, and CREB were correlated with increased occupancy of RNA polymerase II at two loci proximal to the TSS following NMDA receptor stimulation. These temporal changes in promoter occupancy could occur thousands of base pairs 5′ of the TSS, suggesting a mechanism that produces a three-dimensional complex, allowing the distal sites to be placed in close proximity to what will become the TSS of *Bdnf* to regulate transcription.

Epigenetic regulation of gene expression on memory and response to stress: impact of p300/CBP associated factor (PCAF) histone acetyltransferase

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Encoding of new information into long-term memory requires the expression of new genes and active regulation of gene transcription. Particularly, CREB binding protein (CBP) or p300/CBP associated factor (PCAF) act as recruiters for components of gene transcription and as histone acetyltransferases. Chromatin remodeling, through histone acetylation and methylation, is also involved in the physiological response to stress or antidepressants. In particular, regulation of brain-derived neurotrophic factor (BDNF) gene expression has been described through histone H3 acetylation. In this study, we characterized the memory abilities and response to stress and antidepressants of PCAF knock-out (KO) mice. At 2 months of age, PCAF KO animals developed mainly short-term memory deficits (spontaneous alternation, water-maze working memory and object recognition) without long-term memory impairments (no passive avoidance and water-maze reference memory deficits). KO mice presented enhanced c-Fos activation and morphological alterations in the hippocampus. The cognitive deficits evolved with age to long-term memory deficits, and appeared due to both post-natal developmental and functional deficits. KO mice showed an exaggerated response to acute stress, measured by increased immobility during forced swimming and increased freezing during conditioned fear stress. Increased plasmatic corticosterone and ACTH levels were measured vs wild-type animals in basal and stress conditions. PCAF KO mice showed decreased hippocampal and hypothalamic BDNF mRNA and protein levels and increased hypothalamic TrkB-FL mRNA levels. Moreover, the BDNF secretion enhancer cysteamine reversed forced swimming immobility in KO mice. PCAF KO mice thus show a hyper-responsiveness to acute stress and hyper-activation of the HPA axis, partly due to deregulation of BDNF and TrkB receptor gene expression resulting in lower regulatory activity of BDNF system.

Structure and regulation of BDNF expression in rodents and human: similarities and differences

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Brain-derived neurotrophic factor (BDNF), a member of the nerve-growth factor family of neurotrophins, has important roles in the physiology of nervous system. We have re-examined the structures and alternative promoters of mouse, rat and human BDNF genes, the expression patterns of alternative transcripts, and introduced a new numbering system for mammalian BDNF exons. The overall organization of human and rodent BDNF genes is very similar. Some differences are present, though. Most importantly, antisense non-coding antiBDNF RNAs are transcribed in the human BDNF gene locus, but not in rodents, displaying partially overlapping expression pattern with BDNF. We have demonstrated the presence of BDNF-antiBDNF RNA duplexes in vivo in adult human brain and studied regulation of BDNF expression by antiBDNF in human cell-lines. We have also studied the *cis*- and *trans*-factors involved in neuronal activity-mediated induction of human and rodent BDNF gene transcription. Our results show that mechanisms of regulation for rodent and human BDNF genes differ substantially.

Epigenetic mechanisms mediating the long-term impact on behavior of the social environment in early life

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The programming of the genome is accomplished by the epigenome. Two elements of epigenomic control are chromatin modification and DNA methylation. Epigenetic patterns are generated during cellular differentiation by a highly programmed and organized process. Nevertheless, they are dynamic and responsive to the environment

especially during the critical periods of gestation and early life as well as later in life. This sensitivity of the epigenetic machinery to the environment offers a conduit through which the environment can sculpt the genome and have a long-term impact on behavior. We will discuss three studies from rat, rhesus monkeys and human delineating the signature of maternal care and social adversity on the epigenome. We used high-density oligonucleotide microarrays combined with methylated DNA immunoprecipitation to map the response of the "methylome" "acetylome" and "transcriptome" in different brain regions as well as T cells and whole blood in adults exposed to social adversity early in life and controls. Our preliminary results indicate a wide signature of early life maternal care and social positioning on the methylome. Our data also suggest evolutionary conservation of the response and its co-clustering in defined genomic regions.

Exocytotic release of amino acids from astrocytes

Exocytotic release of glutamate from astrocytes

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Astrocytes can exocytotically release the transmitter glutamate. Increased cytosolic Ca^{2+} concentration is necessary and sufficient in this process. The source of Ca^{2+} for the Ca^{2+}-dependent exocytotic release of glutamate from astrocytes predominately comes from endoplasmic reticulum (ER) stores. An additional source of Ca^{2+} in this process comes from the extracellular space; canonical transient receptor potential 1 protein, which form channels that are activated by depletion of internal Ca^{2+} stores allow Ca^{2+} entry from the extracellular space. Mitochondria can modulate cytosolic Ca^{2+} levels by affecting two aspects of the cytosolic Ca^{2+} kinetics in astrocytes. They play a role in immediate sequestration of Ca^{2+} during the cytosolic Ca^{2+} increase in stimulated astrocytes as a result of an increased cytosolic Ca^{2+}. As cytosolic Ca^{2+} declines due to activity of pumps, such as the smooth ER Ca^{2+}-ATPase, free Ca^{2+} is slowly released by mitochondria into cytosol. Taken together, ER, extracellular space and mitochondria, can vary concentration of cytosolic Ca^{2+} which in turn can regulate Ca^{2+}-dependent vesicular glutamate release from astrocytes. Vesicular glutamate transporters (VGLUTs) are responsible for vesicular glutamate storage and exocytotic glutamate release from astrocytes. Over-expression of individual isoforms of VGLUTs in solitary astrocytes shows that VGLUT-3, but not VGLUT-1 and -2, enhances glutamate release from astrocytes without affecting their intracellular Ca^{2+} increase. Inhibition of glutamine synthetase activity by L-methionine sulfoximine in astrocytes, which raises cytoplasmic glutamate levels, greatly increases the exocytotic glutamate release. Taken together, VGLUTs and cytoplasmic glutamate levels in astrocytes regulate exocytotic release from these cells.

From genes to proteins to understand human genetic diseases

Maternal polymorphisms in folate metabolizing genes and the risk of down syndrome offspring

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The present study aimed at evaluation of *MTHFR* 677C/T and 1298A/C polymorphisms in the *MTHFR* gene as maternal risk factors for DS. Forty two mothers of proven DS outcomes and 48 control mothers with normal offspring were included. Complete medical and nutritional histories for all mothers were taken with special emphasis on folate intake. Folic acid intake from food or vitamin supplements was significantly low (below the recommended daily allowance) in the group of case mothers compared to control mothers. Frequencies of *MTHFR* 677T and *MTHFR* 1298C alleles were significantly higher among case mothers (32.1 and 57.1%, respectively) compared to control mothers (18.7 and 32.3%, respectively). Heterozygous and homozygous genotype frequencies of *MTHFR*at position 677 (CT and TT) were higher among case mothers than controls (40.5 vs. 25% and 11.9 vs. 6.2%, respectively) with an odds ratio of 2.34 [95% confidence interval (CI) 0.93–5.89] and 2.75 (95% CI 0.95–12.77), respectively. Interestingly, the homozygous genotype frequency (CC) at position 1298 was significantly higher in case mothers than in controls (33.3 vs. 2.1% respectively) with an odds ratio of 31.5 (95% CI 3.51–282.33) indicating that this polymorphism may have more genetic impact than 677 polymorphism. Heterozygous genotype (AC) did not show significant difference between the two groups. We here report on the first pilot study of the possible genetic association between DS and *MTHFR* 1298A/C genotypes among Egyptians. Further extended studies are recommended to confirm the present work.

Phenylketonuria and tyrosinaemia: a review of 12 years experience of investigations in our laboratory practice

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Several inborn disorders of aromatic amino acid metabolism are well-defined. The clinical characteristics and the associated enzymatic blocks permit them to be easily recognized. The metabolism of phenylalanine (Phe) and tyrosine (Tyr) is still a brilliant example that biochemical defect can occur in each step of this single pathway. The most frequently encountered disorders are phenylketonuria (PKU) with other hyperphenylalaninaemic variants and the various forms of tyrosinaemia. However, if early diagnosis,

immediate onset of treatment and carefully controlled diet in PKU enable the infant to grow up normally, the prognosis of tyrosinaemia still very poor.

Since 1996, we had to answer several requests for diagnosis of these metabolic diseases. One hundred and six PKU and ten Hepatorenal tyrosinaemia (TH) have been identified among 1,768 patients (0–13 years old) by measuring plasma Phe and Tyr concentrations. Preliminary urinary tests were also used.

Unfortunately, at date, neonatal blood screening for PKU is not effect in our societies. Only twelve neonates cases have been detected in ten known families. The diagnosis of other cases (94) was established late in children with mental retardation. Since treatment by selective restriction of Phe has intake, we continue to monitor this indispensable amino acid.

For TH cases, seven were presented with hepatic failure and cirrhosis at 4 and 11 months of age. The others three were detected by clinicians before 3 months of age, two because hypoglycemia and the other with hepatomegaly.

Tyr measurement was assessed. However, hypertyrosinaemia is non discriminant feature of the TH. It lacks specificity because other disorders in Tyr catabolism can lead to increased Tyr. In order to reduce false-positive results α-fetoprotein levels were determined and the biochemical evidence of renal tubular dysfunction was confirmed.

Glutamate, its transporters and antioxidant defenses

Protective role of system x_c- in vivo against oxidative stress

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Cystine/glutamate exchange transporter (system x_c-) consists of xCT and 4F2 heavy chain, of which the former functions as the carrier protein. In mammalian cultured cells, this transporter plays pivotal roles for maintaining the intracellular glutathione level and extracellular cystine/cysteine redox state. By various stimuli including oxidative stress, xCT is strongly induced and intracellular glutathione is increased. Thus, xCT is thought as one of the cellular defense systems against oxidative stress. However, it is not clear whether xCT functions as the cellular defense system also in vivo. Recent studies have suggested that xCT causes some unfavorable outcomes under certain conditions. We have therefore investigated the role of xCT in vivo by comparing xCT-deficient mice with the wild-type mice against paraquat-induced oxidative stress. The viability of xCT-deficient mice by administration of 45 mg/kg paraquat (i.p.) was significantly lower than that of the wild-type mice. Under these conditions, the glutathione level in the lung of xCT-deficient mice was significantly lower than that of wild-type mice. By the administration of paraquat, xCT mRNA in the lung of the wild-type mice was strongly induced, while the expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme for glutathione synthesis, was unchanged in xCT-deficient and wild-type mice. These results indicate for the first time that xCT contributes to sustaining glutathione level and plays the protective role also *in vivo* under certain conditions such as the exposure to severe oxidative stress.

Crucial role of EAAC1, a member of glutamate transporter family, in cysteine uptake and glutathione level in the brain

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Glutathione (GSH) is a tripeptide comprised of glutamate, cysteine and glycine. Cysteine is the rate-limiting substrate for GSH synthesis within neurons. Most neuronal cysteine uptake is mediated by sodium dependent excitatory amino acid transporter system, known as excitatory amino acid carrier 1 (EAAC1). EAAC1-null mice have reduced neuronal GSH levels and, with aging, develop brain atrophy and behavioral changes. Hippocampal neurons of EAAC1−/− mice show reduced GSH content, increased oxidant levels and increased susceptibility to oxidant injury. These changes were reversed by treating the EAAC1−/− mice with *N*-acetylcysteine (NAC), a membrane-permeable cysteine precursor. EAAC1−/− mice also show reduced the number of dopaminergic neurons in the substantia nigra, with aging. An experimental model of Parkinson's disease, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice, showed reduced motor activity, reduced GSH contents, EAAC1 translocation to the membrane, and increased levels of nitrated EAAC1. These changes were reversed by pre-administration of NAC. Pretreatment with hydrogen peroxide, L-aspartic acid β-hydroxamate, or 1-methyl-4-phenylpyridinium (MPP^+) reduced the subsequent cysteine uptake in midbrain slice cultures. These findings suggest that EAAC1 dysfunction impairs neuronal GSH synthesis, via reducing cysteine uptake, to cause neurodegenerative diseases.

Regulation of neuronal glutathione level by GTRAP3-18 via EAAC1 in neurons in vitro and in vivo

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Glutathione is an essential reductant which protects cells and is reduced in neurodegenerative disorders such as Parkinson's and Alzheimer's diseases. Neurons rely mainly on a cysteine transporter termed excitatory amino acid carrier 1 (EAAC1). Regulation of EAAC1 for intracellular glutathione content was investigated using HEK 293 cells as a model system, primary cultured mouse neurons and mouse brain in vivo. In HEK 293 cells, the glutathione content decreased when the GTRAP3-18 protein level at the plasma membrane was increased by methyl-beta-cyclodextrin, rendering the cells more vulnerable to oxidative stress. Intracellular glutathione increased when the GTRAP3-18 protein level at the plasma membrane was decreased by antisense oligonucleotides, rendering the cells more resistant to oxidative stress. Furthermore, the increase in glutathione content produced by stimulating protein kinase C, a translocator and activator of EAAC1, was inhibited by an increase in cell surface GTRAP3-18 protein. In the mouse brain as well as in primary cultured neurons, glutathione increased when the GTRAP3-18 protein level was decreased by genetic manipulations such as siRNA, whereas glutathione decreased when GTRAP3-18 was

increased by methyl-beta-cyclodextrin. Furthermore, glutathione contents that had been increased by a translocator and activator of EAAC1 were suppressed by increased cell surface GTRAP3-18 protein. Our results demonstrate GTRAP3-18 to dominantly and negatively determine the intracellular glutathione contents in neurons.

Glial versus Neuronal glutamate transporters in maintenance of antioxidant defenses

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Glutamate transporters (EAATs) clear extracellular glutamate and provide substrates intracellularly for synthesis of the antioxidant tripeptide gamma-glutamyl-cysteinyl-glycine (glutathione, GSH), transporting both glutamate and cystine (glial subtype)/cysteine (neuronal subtype). We had previously found in differentiated pure striatal astrocyte cultures that L-trans-pyrrolidine-2,4-dicarboxylate (PDC), a substrate inhibitor of EAATs, induces oxidative cell death through depletion of a specific intracellular pool of glutamate normally fuelled by transport and crucial to maintain GSH levels. We had called this novel form of toxicity the "low intracellular glutamate-linked oxidative toxicity" or LIGO toxicity to underscore its differences with the classically described mechanisms linked to high extracellular glutamate, i.e., excitotoxicity and oxidative glutamate toxicity. Here, we investigated the consequences of EAAT dysfunction using PDC on primary embryonic mesencephalic cultures. PDC treatment triggers early and sustained increase in extracellular glutamate levels and neuronal death through NMDAR-mediated excitotoxicity. PDC also induces delayed glutathione depletion, ROS production, and oxidative processes are involved in the death of astrocytes and dopamine neurons while non-dopamine neurons are less affected. Moreover, PDC-induced oxidative stress potentiates NMDA toxicity in dopamine neurons only. Finally, the PDC-induced death of dopamine neurons was found to be direct, being protected by cysteine, while that of non-dopamine neurons was found to be secondary to astrocyte demise, astrocytes and non-dopamine neurons being protected by cystine. Altogether, these results suggest that PDC selectively renders neurons more vulnerable to NMDAR-dependent excitotoxicity through different mechanisms depending on the neuronal phenotype. As glutamate does not mimic the PDC effect, the way glia exacerbates NMDAR-dependent excitotoxicity remain to be determined.

Effects of gliotoxic amino acids on antioxidant defence mechanisms in astrocytes

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The gliotoxins are a group of glutamate analogues that are toxic to astrocytes. Examples include L-α-aminoadipate (L-αAA), L-serine-*O*-sulphate and *β*-*N*-oxalylamino-L-alanine (L-BOAA). Gliotoxins are either inhibitors and/or substrates the high-affinity glutamate transporters and the system x_c^- exchanger.

Pre-incubation of C6 glioma cells with a sub-toxic concentration of L-α-aminoadipate or L-serine-*O*-sulphate (400 μM for 20 h), followed by addition of 1 mM ^{13}C-glycine, caused a significant increase in ^{13}C-labelled glutathione. In primary astrocyte cultures, L-serine-*O*-sulphate caused a significant reduction in de novo synthesis of labelled glutathione from ^{13}C-glycine after 4 h.

In contrast, incubation of C6 glioma cells with L-BOAA or L-αAA (400 μM), decreased the glutathione concentration after 24 h, as determined by fluorimetric analysis. Both gliotoxins caused a comparable reduction in intracellular cysteine, with no loss in cell viability. Addition of *N*-acetylcysteine (400 μM) abolished the reduction in GSH by L-BOAA, but not L-αAA. Co-incubation of C6 cells with L-BOAA and 1 mM propargyl glycine, an inhibitor of cystathionine-γ-lyase, potentiated the reduction in GSH caused by L-BOAA. Pre-incubation of C6 glioma cells with L-BOAA or L-αAA caused a significant increase in expression of cystathionine-γ-lyase.

It is concluded that these gliotoxins cause a reduction in GSH in C6 glioma cells due to loss of intracellular cysteine and inhibition of GSH synthesis via the γ-glutamyl cycle. However, prolonged incubation of the cells in the presence of gliotoxin leads to an up-regulation of the trans-sulphuration pathway, leading to an augmentation in de novo synthesis of the antioxidant.

Microglial self defence mediated through GLT-1 and glutathione

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Microglia are considered as the immunocompetent cells of the central nervous system (CNS). Being the first line of defence, they have prominent roles in monitoring the CNS homeostasis and can rapidly and specifically react to any disturbances or pathological stimuli. As such, it is of importance that microglia are able to survive during the harsh conditions of a pathological event, often leading to high concentrations of free glutamate and oxidative species. Microglia are able to express Na^+-dependent high affinity glutamate transporters under pathological conditions. We have shown that the neuroinflammatory cytokine TNF-alpha can induce GLT-1 expression in microglia and increase the activity of the X_C^--system, leading to functional uptake of glutamate and better conditions for synthesis of glutathione, a major antioxidant. Using ^{3}H-glutamate, we have shown that microglial glutamate uptake is directly coupled to increased synthesis of glutathione as well as for fuelling the intracellular glutamate stores to provide uptake of cystine for incorporation into glutathione. Increased levels of glutathione can provide microglia with a self defence against reactive oxidative species. Being antiviral, glutathione also provides microglia with a defence against viral infections. In response to herpes simplex virus infections, microglia release TNF-alpha and up-regulate their GLT-1 expression in order to provide means for increased glutathione synthesis and thus an increased viral resistance compared to astrocytes or neurons. In summary, our results show that microglial glutamate uptake through GLT-1 is coupled to glutathione synthesis and increased resistance to oxidative stress and viral infections.

Neurotrophins differently regulate glutamate transport in macrophages and astrocytes

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Glutamate and related compounds may act as excitotoxins and participate in neuronal damage in a variety of neurological disorders. Neurotrophins are involved in CNS development, and are now recognized for their actions in mature CNS and pathology, including interferences with glutamate signalling. Indeed, recent studies showed that neurotrophins protect neurons against glutamate excitotoxicity. On the other hand in neurons, extracellular glutamate induces BDNF expression. Likewise, neurotrophin signalling and inflammation may influence each other, as suggested by the expression of NGF in activated perivascular macrophages in HIV encephalitis. Astrocytes are key cells in glutamate homeostasis, a function that macrophages and microglia also support under inflammatory conditions, where astrocyte function is disturbed. We therefore evaluated in vitro the effects of neurotrophins on glutamate metabolism in human macrophages, embryonic and adult astrocytes.

Our results revealed different neurotrophin response profiles related to cell type and development stage. Macrophages have their glutamate clearance capacity decreased by one half by NT3 while embryonic astrocytes have their glutamate uptake capacity shortly (24 h) reduced by one fifth by every neurotrophins. Alternatively, adult astrocytes have their glutamate uptake capacity greatly increased by BDNF, associated with increased EAAT1 gene expression. Surprisingly, both cell types have unchanged intracellular glutathione content under neurotrophin stimulation. These data suggest that BDNF expression may constitute an adaptive response to extracellular glutamate in adult astrocytes and therefore a potential way to protect against excitotoxicity in the mature brain. They also suggest fine regulation of GSH permitting to maintain constant levels despite regulated glutamate uptake by EAAT.

Cooperative action of EAATs and system x_c^- in maintenance of antioxidant defenses

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Oxidative stress plays a role in various neurodegenerative diseases. One of the major small molecule antioxidants in the brain is the tripeptide glutathione. The limiting amino acid for glutathione synthesis is cysteine, which can either imported into cells as cysteine or its reduced form, cystine. The major transporter for cystine is the cystine/glutamate antiporter system x_c^-. It consists of the specific subunit, xCT, and a heavy chain, 4F2hc. Cystine import via system x_c^- is inevitably coupled to glutamate release from cells in a 1:1 ratio. Furthermore, glutamate is competes with cystine uptake via system x_c^-. Intracellular glutamate concentrations are considerably higher that extracellular glutamate. This gradient provides the energy for cystine uptake via system x_c^-.

In the brain, extracellular glutamate levels are kept low by high-affinity excitatory amino acid transporters (EAATs). Thereby, EAATs might reduce the inhibitory effect of extracellular glutamate on cystine uptake. Furthermore, EAATs refuel the intracellular glutamate pools. Thus, these indirect effects are the proposed mechanisms by which EAATs support cystine uptake via system x_c^- and glutathione synthesis.

Furthermore, the cooperative action of EAATs and system x_c^- might not only necessary for optimal x_c^- function but also might prevent deleterious consequences of an upregulated system x_c^- activity with subsequent risk of excitotoxicity by increased glutamate release. Thus, compounds that induce both xCT and EAATs might be powerful neuroprotectives. Ceftriaxone, a beta-lactam antibiotic, which has recently been identified as an inducer of EAAT2, also induces system x_c^- in neuronal cells, motorneurons and astrocytes.

The glutamate/cystine antiporter system x_c^-: more than a mere supplier of cysteine for glutathione and protein synthesis

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Burkitt's lymphoma (BL) cells are highly sensitive towards oxidative stress and rapidly undergo cell death when cultured at low cell densities. The underlying mechanism is impaired uptake capacity for L-cystine, the oxidised and predominant form of L-cysteine in the extracellular space. L-cysteine is the substrate-limiting step in glutathione (GSH) synthesis. Starting to dissect the cross-talk and redundancy between major redox systems, we stably expressed xCT light chain of the glutamate/cystine antiporter in BL cells. Overexpressing BL cells became highly resistant to oxidative stress and GSH depletion. Contrary to our expectations, GSH levels were not altered in overexpressing versus control cells. By contrast, xCT overexpressing cells displayed increased intracellular and strongly boosted extracellular cysteine levels, which efficiently prevented cell death induced by oxidative stress. Our findings establish that system x_c^- sustains the cystine/cysteine redox cycle, which acts independently of intracellular GSH concentrations. Results from studies involving xCT overexpression in GSH-deficient cells and xCT gain of function studies in the mouse will be discussed.

Glutamate/cystine antiporter system x_c^- in regulating pigment biogenesis, cell proliferation and invasion, neuronal function

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System x_c^- is an anionic amino acid transport system highly specific for cystine and glutamate. This antiporter is composed of two protein subunits, xCT and 4F2 heavy chain. xCT mediates the transport activity in the plasma membrane and regulates intracellular GSH levels. Most studies have been focused on the role of xCT in regulating cell proliferation through ROS signaling. We have previously identified an xCT null mutation in subtle gray (*sut*) mouse which reduces greatly the pheomelanin levels in cultured melanocytes and hairs of *sut* mice due to inefficient incorporation of cysteine into cysteinyldopa, a precursor of pheomelanin. We further characterized

that cultured cells from *sut* mice grew very poorly without supplement of beta-mercaptoethanol (BME) in the medium. Our results show that ROS is elevated in cultured *sut* melanocytes. Death of cultured *sut* cells is likely caused by apoptosis through JNK activation. Furthermore, suppression of xCT activity by sulfasalazine (SASP) inhibits the cell proliferation and clone formation of a human esophageal squamous cell carcinoma cell line (KYSE150). SASP significantly inhibited both cell invasion of KYSE150 in vitro and its experimental metastasis in nude mice. Further study revealed that the upregulation of caveolin-1 and inhibition of tumor cell invasion by xCT disruption (SASP or siRNA) were mediated by ROS-induced p38 MAPK activation. These results first established the dual role of xCT in carcinogenesis and cancer metastasis. Interestingly, xCT is defined as an entry receptor for HHV-8, a causative virus for Kaposi sarcoma (KS). It is postulated that xCT may have a dual role in the formation of KS by involving both virus infection and oncogenesis. Finally, we will discuss possible roles of xCT in regulating oxidative stress and basal level of glutamate in *sut* neuronal cells which may lead to abnormal behaviors in *sut* mice.

Glycoproteomics

GlycoHepatoTest: discovery, validation and translation to the clinical laboratory setting

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Chronic liver disease is currently assessed by liver biopsy, a costly and rather cumbersome procedure that is unsuitable for patient monitoring. Therefore, non-invasive markers are needed that could be frequently used to follow up the fibrosis progression, detect cirrhosis at an early (clinically compensated) stage and detect hepatocellular carcinoma in cirrhosis patients.

We have investigated whether the profiling of the *N*-glycan mixture derived from total serum protein can be used for these purposes (GlycoHepatoTest). To allow for relevant clinical diagnostic studies on several hundred samples and clinical implementation, we have optimized the sample preparation method to only require a PCR thermocycler and a capillary DNA-sequencer. The protocol only involves pipetting and incubation steps and is completed in less than 1 h. Present efforts are directed at implementing the analysis on low-cost electrophoretic analyzers to further ease clinical use. In three multicenter clinical trials, GlycoHepatoTest was benchmarked against current clinical practice (histological scoring of biopsy) and existing serum biomarkers for liver disease. For liver fibrosis monitoring, GlycoFibroTest increases gradually with METAVIR fibrosis stage from F1 onwards. For the diagnosis of early-stage liver cirrhosis, GlycoCirrhoTest together with HA or Fibrotest allows to diagnose about 60% of the patients with >95% specificity, which was previously impossible. This allows to enlist these patients for more intense HCC screening. For patients with advanced liver cirrhosis, GlycoHCCTest together with AFP allows almost double sensitivity than AFP alone, while maintaining >90% specificity. Moreover, the GlycoHCCTests score increases with more advanced tumor stage.

Pharmacological significance of the glycosylation of immunoglobulins

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Examples of the influence of the structure of carbohydrate units attached to immunoglobulin on its pharmacological efficacy will be presented. The best known case is the substantial enhancement of antibody-dependent cellular cytotoxicity (ADCC) by non-fucosylated antibodies. While over 90% of natural IgG *N*-glycans bear a fucose residue, the interaction of Fc-gamma receptors with non-fucosylated antibody displays a much higher affinity. The higher ADCC, however, is apparently obtained at the expense of some reduction of the complement dependent cytotoxicity (CDC). CDC in turn may also be influenced by galactosylation. Given the important role of ADCC for tumor cell killing there is great interest in approaches for the production of non-fucosylated recombinant antibodies. Alternatively, a similar effect on ADCC may be achieved by amino acid substitutions in the Fc-domains. A recent paper even claims that changes in protein sequence can compensate for the virtual loss of Fc-effector functions associated with non-glycosylation of the Fc-region.

Smaller points are the lowering of IgG serum half-life by oligomannosidic *N*-glycans and the severe side effects of alfa-galactosylated glycans, which are found on antibodies produced in mouse cell lines.

Finally, the recent association of antibody sialylation with the anti-inflammatory effect of high intravenous doses of IgG will be discussed.

Glycoproteomics of human milk: an antimicrobial defense mechanism?

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Human milk contains a highly potent mixture of protective agents such as free sugars, antibodies and anti-microbial proteins constituting an innate immune system, whereby the mother protects her infant from enteric and other diseases. Human milk free sugars have been reported to inhibit microbial pathogenesis in various in vivo, ex vivo and in vitro experimental systems however the changes which occur to specific sugar structures attached to glycoproteins in milk over the course of lactation have not been investigated.

Glycoproteins are contained in the soluble fraction of milk as well as in the milk fat globule membrane (MFGM)[1] which surrounds and solubilises the lipid. The oligosaccharide structures expressed on these proteins were found to be blood group independent of the individual, so that it is possible that the protection offered by these sugars in milk is common to all women. The N- and O-linked glycan phenotype, both of the total glycoprotein complement as well as of specific glycoproteins, of milk from five different individuals during the course of infant breastfeeding will be presented. The structures of those protein-attached glycans which are recognized by human gut pathogens will be described. These oligosaccharide moieties present on the milk glycoproteins, with homology to epithelial mucus cell surface pathogen receptors in the stomach and intestine, may inhibit

infection by competitively binding with the pathogens and clearing them from the infant gut. This knowledge offers the opportunity to design glycoconjugates that may be added to infant milk formula to help protect infants from endemic pathogens.

Homocysteine versus hydrogen sulfide A production of hydrogen sulfide and its release in the brain

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Hydrogen sulfide (H_2S) is a synaptic modulator as well as a neuroprotectant. Currently, pyridoxal-5′-phosphate (PLP)-dependent cystathionine β-synthase (CBS) is thought to be the major H_2S producing enzyme in the brain. We recently found that brain homogenates of CBS knockout mice even in the absence of PLP produce H_2S at levels similar to those of the wild-type mice, suggesting the presence of another H_2S producing enzyme. 3-Mercaptopyruvate sulfurtransferase (3MST) in combination with cysteine aminotransferase (CAT) produces H_2S from cysteine. In addition, 3MST is localized to neurons and the levels of bound sulfane sulfur are greatly increased in the cells expressing 3MST and CAT but not increased in cells expressing functionally defective mutant enzymes. Bound salfane sulfur releases H_2S in neurons and astrocytes in the presence of physiological concentrations of endogenous reducing substances glutathione and cysteine. Alkalinization of the cytoplasm is required for effective release of H_2S from bound sulfane sulfur, and this condition is achieved in astrocytes by high concentrations of extracellular K^+ that are normally present when nearby neurons are excited. These data present a new perspective on H_2S production, storage and release in the brain.

Homocystine causes mitophagy; protection by hydrogen sulfide

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Hyperhomocysteinemia (HHcy) is frequently observed in chronic heart failure (CHF) resulting in oxidative stress, interstitial and perivascular fibrosis that increases the propensity for mitochondrial mitophagy. Hcy is metabolized in the body to produce an endogenous gaseous substance, hydrogen sulfide (H_2S). The potential role of H_2S in mitophagy remains untouched. Therefore we hypothesized that the HHcy increased calcium overload, in part closing K_{ATP} channels, oxidative molecule, martix protein and mitochondria dysfunction in myocardial infarction mice heart. Wildtype (WT), WT + myocardial infarction(MI),WT + NaHS (NaHS is a H_2S donor, 30 μmole/L in drinking water) and MI + NaHS mice were used. The mitochondria were isolated from heart tissue and analyzed for Hcy-induced mitochondria damage, function and the cardioprotective role of this gaseous substance. The results suggested that levels of mitochondrial Hcy increased and H_2S decreased in post MI. Levels of CBS, CSE and MTHFR were decreased. SAHH level was increased. Mitophagy markers, LC-3 and p62, levels revealed mitochondrial mitophagy in MI hearts. The H_2S mitigated this mitophagy. The MMP-2, -9, TIMP-2 and 3 expression were increased in MI hearts. There was a disruption of mtCxn43 and mtTJP in MI hearts. Mitochondrial membrane potential was high, oxygen consumption was low, and the levels of ATP were decreased in MI hearts. In vivo echocardiography, pressure volume loop data, barium contrast X-ray angiography revealed increase in collateral flow in MI hearts after H_2S treatment. The findings of this study may open new avenues for future investigations of therapeutic potential of this novel gaseous substance in HHcy associated mitophagy.

Metalloproteins

Transition metal homeostasis in bacteria as a flow equilibrium of uptake and efflux processes

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To understand bacterial heavy metal resistance, the function of several groups of different metal efflux systems and their physiological interplay was analyzed in *Escherichia coli* and the metal-resistant bacterium *Cupriavidus (Wautersia, Ralstonia, Alcaligenes) metallidurans*. RND (resistance, nodulation, cell division) systems form a first line of defense. Evidence from several RND systems indicates that RND systems in vivo protect the periplasm against superfluous heavy metal cations. CDF (cation diffusion facilitators) and P-type ATPases transport toxic metals from the cytoplasm across the cytoplasmic membrane, forwarding them to the RND systems. Contribution of at least one of the two the chromosomal Zn/Cd-exporting CPx-type ATPases of *C. metallidurans* is essential for full cadmium resistance, but not for full zinc resistance. Three groups of CDF proteins can be differentiated. Each group contains transporters with a broad substrate specificity, which is respectively centered around the central substrates Fe(II), Zn(II) or Co(II). The function of CDF proteins is essential to obtain cobalt resistance in *C. metallidurans*, which demonstrates that RND proteins are unable to detoxify substrates from the cytoplasm, but gather them in the periplasm instead. The contribution of the RpoE-sigmulon to metal homeostasis in *E. coli* demonstrates the importance of this cellular compartment for metal resistance.

Structure-function relationships of hydrogenases

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Hydrogenases are enzymes present in many micro-organisms where they catalyze the oxidation of molecular hydrogen or the reduction of protons. The three known types of hydrogen-metabolizing enzymes are [NiFe]-, [FeFe]- and [Fe]-hydrogenases. They share a $Fe(CO)_x$ unit in their active sites that is most likely involved in hydride binding. Because of their complexity, hydrogenases require a maturation machinery that involves several gene products. Depending on the enzyme, they include metal ion transport, synthesis of CN^- (and maybe CO), formation and insertion of a $FeCO(CN^-)_2$, $Fe_2(CO)_2(CN^-)_2$ or $Fe(CO)_2$ unit in the apo form, synthesis of a small dithiolate-containing molecule (FeFe-hydrogenase) or insertion of nickel and proteolytic cleavage of a C-terminal stretch (NiFe-hydrogenase). Because the active sites of NiFe- and FeFe-hydrogenases are buried in the structure, electron and proton transfers are required between their sites and the

molecular surface. The former is mediated by either three or one Fe/S cluster, depending on the enzyme. Proton transfer pathways have been postulated based on both the structural analyses and theoretical calculations. Most hydrogenases are inactivated when exposed to oxidizing conditions, such as the presence of O_2. Understanding this process is of bio-technological interest for the H_2 production by photosynthetic organisms and the design of bio-fuel cells.

Hijacking transferrin bound iron: the roles of TbpA/B, FbpA and an exogenous anion in moving iron to the cytosol

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Ferric binding protein A is the central component of a highly conserved, three protein ABC-transporter (FbpABC). FbpA sequesters free Fe^{3+} from the outer membrane receptor (TbpA/TbpB), which hijacks Fe from transferrin docked at the outer membrane, and, transports iron across the periplasm to the cytoplasmic membrane receptor (FbpB/C). This system is highly conserved in the obligate human pathogens *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis,* where the essential Fe nutrient is bound to human serum transferrin. The pathogenicity of each these bacteria is directly related to the efficiency of the Fe acquisition process and consequently a detailed understanding of the mechanism is of interest.

We will present biophysical and microbiological data relevant to the mechanism whereby Fe is tightly sequestered at the inner face of the outer membrane receptor TbpA/B and transported in the presence of an exogenous anion across the periplasm to the cytoplasmic membrane receptor FbpB/C. We will present kinetic and thermodynamic data to suggest that the exogenous anion plays a key role in both the iron sequestration and release processes, and further that FbpA may be viewed as an Fe-anion-complex transporter, rather than an Fe transporter. Site directed mutagenesis will be used in conjunction with variations in exogenous anions to elucidate the stepwise processes involved in Fe sequestration. A redox hypothesis will be presented for the initial step in the release of Fe^{2+} at the cytoplasmic membrane receptor.

Heme fishing by hemophore HasA and transport by the outer membrane receptor, HasR

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Iron, an essential nutrient for many metabolic pathways, is mostly insoluble or tightly sequestered by iron or heme binding proteins. To satisfy their need on iron, several Gram-negative bacteria use a heme uptake system involving an extracellular heme-binding protein HasA, also called hemophore. The function of hemophore is to acquire free or hemoprotein-bound heme and to deliver it to a specific outer membrane receptor, HasR. The heme transport through the receptor is an active process driven by the proton motive force.

HasR of *Serratia marcescens* belongs to the TonB class dependent outer membrane transporters. All the receptors of this family need the energy driven by an inner membrane complex composed of TonB-ExbB-ExbD to internalise their substrate. In this system, a specific TonB like protein, HasB, is dedicated to HasR.

To unravel the mechanism of this active transport by a specific transporter we studied the structure and the molecular interactions of proteins involved in different steps from the substrate recognition to the heme internalisation.

Membrane ferrisiderophore trafficking in bacteria: metal and ligand specificities

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Most bacteria meet their iron requirements by producing and secreting low-molecular mass iron-binding compounds called siderophores. Siderophores provide the cell with iron by solubilizing the ferric ion. The uptake of ferric-siderophores into Gram-negative bacteria involves specific outer membrane transporters and ABC transporters for respectively the translocation across the outer and the inner membrane.

To acquire iron, *Pseudomonas aeruginosa* secretes two major fluorescent siderophore, pyoverdine and pyochelin, and they specific outer membrane transporters, FpvA and FptA respectively. We took advantage of the fluorescence properties of these siderophores to investigate the siderophore and metal specificities of these two iron uptake pathways. Our findings indicate that siderophores are able to chelate many other metals in addition to iron. At the cell surface, a broad metal specificity was shown for FpvA and FptA at the binding stage, and a strong metal selective for the uptake process. Apparently, it is the mechanism of ferrisiderophore translocation across the outer membrane which drives the metal selectivity for this iron uptake pathways.

The fluorescent properties of pyoverdine allowed us to follow as well the fate of this siderophore after its uptake across the outer membrane. An iron reduction is required for the dissociation of pyoverdine-Fe and this dissociation occurs in the periplasm. Pyoverdine is not modified but recycled to the medium, still competent for iron chelation and transport.

The different data obtained will be used to discuss possible transport mechanisms and to highlight relevant features of membrane iron trafficking in Gram negative bacteria.

Structure and dynamics of HasB, a specific TonB like protein, and its interaction with HasR, a heme/hemophore transporter

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Iron, an essential nutrient for many metabolic pathways, is mostly insoluble or tightly bound to iron or to heme binding proteins. To satisfy their need for iron, several Gram-negative bacteria use a heme uptake system involving an extracellular protein HasA, also called

hemophore. The function of hemophore is to acquire free or hemoprotein-bound heme and to deliver it to a specific outer membrane receptor, HasR. The heme transport through the receptor is an active process driven by the proton motive force.

HasR of Serratia marcescens belongs to the class of TonB dependent transporters (TBDT). All the receptors of this family need the energy generated by an inner membrane complex composed of TonB-ExbB-ExbD to internalise their substrates, like heme, vitamin B12, iron siderophore etc. The known 3D structures of two complexes of TBDT with the periplasmic domain of TonB show that the TonB box, a conserved N-terminal region in the transporters, is critical for this interaction. *Serratia marcescens* possesses two TonB-like proteins. One of them, HasB, is specifically dedicated to HasR.

In this work, we present the structural and dynamical properties of the periplasmic domain of HasB (131 residues) studied by heteronuclear NMR. The structure of HasB presents significant differences when compared to that of TonB. The interaction of HasB with the HasR TonB box has also been investigated. The interface region of the complex has been identified. The experiments for the structure determination and the dynamics characterization of the complex are presently underway. The first results show an extensive change in the HasB structure upon binding. These results will allow to better understand the functional differences between TonB and HasB and the specificity of HasB to HasR.

Mitochondrial carriers involved in amino acid transport

Mitochondrial transporters for aspartate and glutamate and related diseases

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The inner membranes of mitochondria contain a large family of nuclear-coded proteins that transport a variety of metabolites, nucleotides and coenzymes across the membranes. In man there are 48 mitochondrial transporters (encoded by the SLC25 genes); about half have been characterized biochemically. Examples are the glutamate carrier and the aspartate/glutamate carrier. In man, both these carriers have two isoforms (GC1/GC2 and AGC1/AGC2, respectively). AGC1 and AGC2 transport L-aspartate, L-glutamate and L-cysteinesulphinate by an obligatory 1:1 exchange; GC1 and GC2 transport only L-glutamate in co-transport with H^+ or in exchange for OH^-. The activity of AGC1 and AGC2 is stimulated by Ca^{2+} acting on the external side of the inner mitochondrial membrane, and this activation results in increased oxidative metabolism and greater ATP formation in the mitochondrion. AGC is essential for the malate-aspartate cycle and for supplying aspartate from mitochondria to cytosol. GC plays an important role in amino acid degradation and specific cell functions such as ureogenesis. Until now, three autosomal recessive diseases have been found to be caused by defects in one of the AGC or GC isoforms. Type II citrullinemia is due to mutations in SLC25A13 coding AGC2. Alterations in SLC25A12 coding the neuron/muscle-specific isoform AGC1 are responsible for global cerebral hypomyelination. Finally, neonatal epileptic encephalopathy with suppression bursts is caused by mutations in SLC25A22 coding GC1.

The mitochondrial ornithine carrier and the HHH syndrome

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The mitochondrial ornithine carrier has two isoforms in man (ORC1 and ORC2) encoded by two different genes SLC25A15 and SLC25A2, respectively. Even though both isoforms catalyze the transport of ornithine, lysine, arginine and citrulline, they differ in many respects, such as substrate specificity, kinetic parameters, tissue distribution and expression levels.

The ORC fulfils the important function of exchanging cytosolic ornithine and intramitochondrial citrulline, and is therefore an essential component of the urea cycle. This conclusion is strengthened by the demonstration that the SLC25A15 gene coding ORC1 is altered in patients affected by the hyperornithinemia–hyperammonemia–homocitrullinuria (HHH) syndrome, an autosomal recessive disorder characterized by growth retardation, periods of lethargy, ataxia, myoclonic seizures and episodes of coma due to hyperammonemia. The co-expression of ORC2 and ORC1 in liver may explain the mild phenotype characteristic of HHH patients, as compared to that caused by defects in any of the other urea cycle enzymes.

As well as in ureogenesis, ORC1 and ORC2 may play a role in cell metabolism under various physiological conditions. For instance the net import of lysine, arginine and histidine into the mitochondria is necessary for the synthesis of intramitochondrially-translated proteins, whereas the efflux of ornithine from the mitochondria may occur in other metabolic processes, such as the biosynthesis of polyamines that are produced from ornithine in the cytosol.

The transport mechanism of mitochondrial carriers based on analysis of symmetry

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The structures of mitochondrial transporters and uncoupling proteins are threefold pseudo-symmetrical, but their substrates and coupling ions are not. Thus, deviations from symmetry are to be expected in the substrate and ion binding sites in the central aqueous cavity. By analyzing the threefold pseudo-symmetrical repeats from which their sequences are made, conserved asymmetric residues were found to cluster in a region of the central cavity identified previously as the substrate binding site. In contrast, conserved symmetrical residues required for the transport mechanism were found at the water–membrane interfaces. Three PX[DE]XX[RK] motifs form a salt bridge network on the matrix side of the cavity, when the substrate binding site is open to the mitochondrial intermembrane space. Three [FY][DE]XX[RK] motifs are present on the cytoplasmic side of the cavity and could form a salt bridge network when the substrate binding site is accessible from the mitochondrial matrix. It is proposed that the opening and closing of the carrier could be coupled to the disruption and formation of the two salt bridge networks induced by substrate binding. The interaction energies of the networks allow members of the transporter family to be classified as strict exchangers or uniporters.

Neurobiology I: nanoneuroscience and neural, glial and axonal protein expression in CNS injury and repair

Carbon nanotubes inhibit depolarization-dependent intracellular Ca^{2+} load and endocytosis in neurons

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The numerous electrical, mechanical and chemical properties that carbon nanotubes (CNTs) possess make them an intriguing material with great promise for application in neuroscience. When water soluble single-walled CNTs (wsSWNTs) were applied to the culture media of hippocampal neurons grown on the permissive substrate polyethylene imine, they caused an alteration in growth and morphology of neurons, notably the extension of neurite length and reduced number of neurites. These wsSWNTs affected intracellular Ca^{2+} dynamics in neurons, reducing the depolarization-dependent influx of Ca^{2+} from extracellular space during cell stimulation. It has been implicated that plasma membrane/vesicular recycling plays a role in the rate of neurite elongation. This process of vesicular recycling can be regulated by an increase in intracellular Ca^{2+} levels due to depolarization of neurons. Indeed, wsSWNTs were able to block stimulated membrane endocytosis in neurons. Consequently, the exocytotic incorporation of vesicles into the plasma membrane is not balanced by the endocytotic retrieval in the presence of wsSWNTs, which could effectively cause the increase in neurite length observed; wsSWNTs' effect on the reduction of the number of neurites could be then a compensatory mechanism to keep the cell surface/volume relatively constant. These findings indicate the exciting possibility that wsSWNTs could be used at the local site of brain injury to enhance outgrowth of selected neurites, thus increasing the chance of "bridging" the injured site.

Nanoparticles influence spinal cord injury induced glial protein expression

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Silicon nanoparticles or silica dust is quite common form of exposure to soldiers engaged in gulf war that may influence their health and brain function. Since nanoparticles from the environment can easily be transported to brain via inhalation it is quite likely that these microfine particles will alter neuronal, glial and endothelial cell functions in the brain leading to neurotoxicity. Previous reports from our laboratory show that exposure of Ag, Cu or Al nanoparticles adversely affect neuronal function following heat exposure indicating that nanoparticles exposure may enhance brain pathology following stressful conditions. Thus, it appears that traumatic injuries to the spinal cord may also be influenced by exposure to nanoparticles.

We exposed rats to silicon nanoparticles (SiO_2, 40–50 nm) by administering them intraperitoneally for 7 days (50 mg/kg) and then subjected them to spinal cord injury (SCI) and examined spinal cord pathology. The results were compared to normal animals subjected to identical SCI. Rats treated with SiO_2 for 7 days did not show any significant alteration in cord pathology. However, subjection of these nanoparticles treated rats to SCI resulted in profound upregulation of glial fibrillary acidic protein immunoreactivity, downregulation of myelin basic protein immunostaining, breakdown of the blood–spinal cord barrier to Evans blue and radioiodine as well as pronounced neuronal distortion and damage. The magnitude these pathological changes in nanoparticles treated animals are more aggravated following SCI as compared to normal rats after trauma. These observations suggest that exposure of nanoparticles enhances the sensitivity of trauma induced neural, glial and myelin damages, not reported earlier. It remains to be seen whether neuroprotective effects of various agents may also vary following SCI in nanoparticles treated animals, a feature currently being investigated in our laboratory.

Nanodrug delivery in spinal cord injury alters neuronal and axonal proteins expression and enhances neurorepair

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The possibility that drugs attached to innocuous nanowires enhance their delivery within the spinal cord and thereby increase their therapeutic efficacy was examined in a rat model of spinal cord injury (SCI). Three compounds AP173 (SCI-1), AP713 (SCI-2) and AP364 (SCI-5) (Acure Pharma, Sweden) were tagged with TiO2-based nanowires using standard procedure. Normal compounds were used for comparison. Spinal cord injury (SCI) was produced by making a longitudinal incision into the right dorsal horn of the T10–11 segments under equithesin anaesthesia. The compounds either alone or tagged with nanowires were applied topically within 5–10 min after SCI. In these rats, blood–spinal cord barrier permeability, edema formation and cell injury were examined at 5 h. Furthermore, expression of c-fos and heat shock proteins (HSP 72kD) was also evaluated using immunohistochemistry. Topical application of normal compounds in high quantity (10 μg in 20 μl) attenuated edema formation, cell injury and reduced BSCB to Evans blue albumin

(EBA) and $^{[131]}$ Iodine. These beneficial effects are most pronounced with AP713 (SCI-2) treatment. Interestingly, when these compounds were administered in identical conditions after tagging with nanowires, their beneficial effects on spinal cord pathology were further enhanced. The nanowired compound AP173 was also found most potent in attenuating c-fos, (a proto-oncogene and neuronal marker), myelin basic protein (MBP, an axonal protein) and heat shock protein (HSP, a stress protein) expression in the spinal cord after SCI. This effect of nanowired compound was most pronounced following SCI as compared to those injured animals that received normal compounds. Interestingly, topical administration of nanowires alone did not influence trauma induced spinal cord pathology or c-fos, MBP or HSP 72 kD expression. Taken together, our results strongly indicate that the nano drug-delivery enhances the therapeutic efficiency of the neuroprotective agents probably by attenuating cellular stress responses effectively.

Nanodrug delivery alters heat shock protein expression in drug dependence and induces neuroprotection

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The possibility that nanodrug delivery of an antioxidant compound H-290/51 will influence spontaneous morphine withdrawal symptoms and induce neuroprotection much more effectively that the parent compound was examined in this investigation in a rat model. Daily administration of morphine (10 mg/kg, i.p.) for 10 days resulted in dependence of rats as seen by loss of analgesic response. On the 11th day, no morphine administration was given. This resulted in profound withdrawal symptoms 24 h after morphine withdrawal. The magnitude and severity of these symptoms were increased further 48 h after withdrawal. Measurement of the blood–brain barrier (BBB) permeability, a measure of perturbed brain fluid microenvironment showed leakage of Evans blue and radioiodine tracers in several parts of the brain in rats showing withdrawal symptoms. Activation of glial cells and expression of heat shock protein (HSP 72 kD) was seen in several regions of the brain and spinal cord of these animals. On the other hand, rats treated with H-290/51 (50 mg/kg, p.o.) either on the first day or second day morphine withdrawal showed much less symptoms, leakage of the BBB and upregulation of GFAP or HSP expressions. These neuroprotective effects were much more pronounced when the H-290/51 was administered using nano delivery techniques. Taken together these observations are the first to suggest that nanodrug delivery significantly attenuates stress symptoms, BBB breakdown, GFAP and HSP expressions caused my spontaneous morphine withdrawal as compared to the normal compound. It appears that oxidative stress associated with the withdrawal symptoms are sufficient enough to induce breakdown of the BBB function and activation of astrocytes and stress protein responses. Nanodrug delivery of antioxidant appears to be the most effective treatment to achieve neuroprotection following morphine withdrawal induced neurotoxicity. Further studies are in progress to see whether nanodrug delivery could also effectively reduce psychostimulants induced brain pathology, a feature currently being investigated in our laboratory.

Nanoparticles exacerbate heme oxygenase expression and brain pathology in heat stress. Neuroprotective effects of antioxidant compound H-290/51

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The possibility that chronic exposure of nanoparticles may exacerbate stress reaction following hyperthermia and induce brain pathology was examined using upregulation of heat shock protein (HSP 72 kD) and hemeoxygenase-2 (HO-2) immunohistochemistry in the rat brain in our model. Engineered nanoparticles from Ag or Cu ($\approx$50–60 nm) were administered (30 mg/kg, i.p.) once daily for 1 week in young male rats and on the 8th day these animals were subjected to 4 h heat stress at 38°C in a BOD incubator. In these animals and stress reaction, blood–brain barrier (BBB) permeability and brain pathology were examined. Subjection of nanoparticle treated rats to heat stress showed exacerbation HSP expression and exhibited much intense upregulation of HO-2 in several parts of the brain showing BBB disruption to protein tracers, brain edema formation and brain damage as compared to normal animals following heat exposure. This effect of enhanced brain pathology in heat stress was most marked in Ag and Cu treated animals. Pretreatment with antioxidant compound H-290/51 either 30 or 60 min before heat stress did not significantly attenuate brain pathology in nanoparticle treated rats. Whereas, administration of nanowired-H-290/51 at identical periods markedly reduced HSP expression, HO-2 upregulation, BBB disruption, brain edema formation and brain pathology in nanoparticles treated heat stressed animals. These results show that chronic nanoparticles treatment exacerbate hyperthermia induced HSP and HO-2 response and enhanced brain pathology. These neuropathological changes were significantly attenuated by nanowired but not normal H-290/51 compound indicating that nanowired drug delivery of H-290/51 is a promising approach to induce neuroprotection in hyperthermia induced brain pathology in nanoparticles exposed animals.

Engineered nanoparticles from metals alter excitatory and inhibitory amino acid neurotransmitter concentrations and induce neuronal, glial and axonal proteins expression and brain pathology

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Influence of nanoparticles on alterations in neural transmission in vivo situations is still unknown. It appears that depending on the magnitude and intensity of exposure to nanoparticles from the environment could affect neuronal transmission and eventually lead to neurotoxicity. This hypothesis was examined in present investigation using systemic administration of engineered nanoparticles from metals, i.e., Al, Ag and Cu ($\approx$50–60 nm) for 7 or 15 days on alterations in excitatory (glutamate and aspartate) and inhibitory (GABA and glycine) neurotransmitters in the rat brain in relation to neurotoxicity. Intraperitoneal (50 mg/kg) administration of nanoparticles for 15 days markedly enhanced the glutamate and aspartate content in the cerebral cortex, hippocampus, thalamus, hypothalamus and cerebellum. On the other hand, a marked decrease in GABA and glycine content were observed in nanoparticles treated animals in these brain regions at this time. These effects on amino acid neurotransmission were most pronounced in animals treated with Ag and Cu nanoparticles as compared to Al treatment. However, changes in excitatory or inhibitory amino acid contents in the brain were less marked in animals that were treated with nanoparticles for 7 days. On day 15, the nanoparticles treated animals avowed marked neuronal, glial and axonal cell injuries. This is evident from presence of many distorted neurons, activation of glial fibrillary acidic protein (GFAP) immunoreactivity and degradation of myelin basic protein immunostaining in several brain areas showing disturbances in amino acid neurotransmission. Taken together these results are the first to show that nanoparticles from metals are able to induce selective and specific increase in excitatory amino acid neurotransmitters and a significant decrease in inhibitory neurotransmission in several brain areas that appears to be instrumental in precipitating neurotoxicity.

New perspectives in peptides- and antibodies-conjugated nanocarriers for brain delivery therapeutic purposes

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The brain delivery of active substances is currently one of the most stimulating challenge due to the inability of conventional medicine to apply effective therapeutic strategies for the treatment of brain pathologies, including neurodegenerative diseases, brain tumors and HIV-related dementia.

The use of nanodevices (ND), such as liposome (Lp), nanoparticles (Np) and solid-lipid nanoparticles (SLNp), has a long-time application as drug delivery systems.

Regarding brain delivery, it is notable that these systems, if not engineered, are totally unable to cross the healthy state BBB; thus, the role of ND surface engineering surely represents the milestone for a promising future application in difficult-to-treat brain pathologies. These ND can be modified with specific ligands or, more generally, substances, able to increase their ability to cross BBB by means of specific mechanisms, such as absorptive-mediated transcytosis or receptor-mediated endocytosis. It is the case of specific peptides, which have been conjugated with polymeric or lipidic nanodevices to allow a more selective drug delivery across the BBB, giving pharmacological evidences of the increase activity.

Moreover, the use of polyclonal or monoclonal antibody (Ab) such as anti-transferrin Ab, surely represents another interesting and efficacious approach, due to the high selectivity of the recognition-reaction with the proper antigen or antibodies; thus nanodevices has been modified with specific antibodies in order to make use of receptor-mediated passages of engineered carriers, thus able to enter the brain and carry drugs in the final correct site of action.

Neurophysiology of inhibitory and excitatory amino acid receptors

Synaptic and extrasynaptic basis for the generation of early network oscillations in the developing cortex

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Spontaneous correlated neuronal activity clearly represents the hallmark of the developing brain since it has been observed in a wide range of peripheral and central structures. In developing cortical structures, several patterns of coherent activity have been described at different stages thus providing a general framework of network maturation. A detailed timely description of network patterns at circuit and cell levels is essential for the understanding of pathogenic processes occurring during brain development. Disturbances in the expression timetable of this pattern sequence will affect network maturation. Early network oscillations (ENOs) are the dominant network pattern in the rodent neocortex for a short period after birth. Using functional multineuron calcium imaging together with single-cell and field potential recordings to clarify distinct network dynamics in rat cortical slices, we have recently shown that the developing somatosensory cortex generates first ENOs then GDPs, both patterns co-existing for a restricted time period. These patterns markedly differ by their developmental profile, dynamics and mechanisms. In this talk, I will focus on the maturation of coherent activity patterns in developing neocortical structures and present the intrinsic and synaptic cellular properties that are unique to the immature neocortex. In particular I will emphasize the critical role played by extracellular glutamate in controlling network excitability and triggering synchronous network waves of activity.

The different roles of AMPA and NMDA receptors on synapse function and plasticity

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In the central nervous system, most excitatory synapses occur on spines, which are small, approximately 1 μm, protrusions of the

dendritic tree. Dendritic spines are heterogeneous in shape and have been classified as mushroom, thin and stubby spines, based on the length of their neck and the size of their spine head. Spines undergo actin-dependent shape changes that occur over a large time scale, ranging from seconds to tens of minutes or even days. It is logical that during development spines undergo motility to form synapses but the physiological role of dendritic spine motility once synapses have been formed in the mature system is unknown. Evidence of the role of glutamate receptors on dendritic spine motility, maintenance and plasticity will be discussed. In particular, chronic blockade of AMPA receptors during excitatory synapse formation does not affect the number or shape of spines whereas chronic blockade of AMPA receptors in mature spines results in the loss of synapses and spines with the formation of more excitatory shaft synapses. Loss of synaptic contacts, in turn, leads to compensatory changes in synaptic transmission and plasticity. In contrast, chronic blockade of NMDA receptors prevents the pruning of dendritic arbors and spines but lowers the threshold for synaptic plasticity. These findings reveal differential roles of glutamate receptors on excitatory synapse formation, maintenance and plasticity.

$GABA_A$ receptors, gephyrin and homeostatic synaptic plasticity

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GABAA receptors are ligand-gated ion channels mediating fast synaptic action of GABA, the main inhibitory neurotransmitter in the CNS. They are major targets for tranquillizers, hypnotics, muscle-relaxants, and anti-epileptic drugs. Regulation of GABAA receptor function and localization is critically dependent on interactions with gephyrin, the main scaffolding protein at GABAergic synapses. Gephyrin forms postsynaptic clusters by autoaggregation, and is traditionally considered to passively anchor receptors at postsynaptic sites.

Synaptic homeostasis describes mechanisms ensuring overall stability of neuronal networks while allowing local changes in synaptic strength and connectivity. Emerging evidence from glutamatergic synapses indicates that synaptic homeostasis is mediated by activity-dependent posttranslational regulation of PSD proteins, which exert direct or indirect influences on glutamate receptor function. While the molecular mechanisms underlying regulation of glutamatergic synapse activity and plasticity are well characterized, little is known about gephyrin regulation at GABAergic synapses. However, recent evidence obtained from in vitro experiments demonstrates that gephyrin clustering is associated with the formation of new GABAergic synapses and actively contributes to synaptic homeostasis.

In this review, we hypothesize that signaling pathways regulating PSD-95 and glutamatergic transmission also participate in the regulation of gephyrin and GABAergic transmission. Specifically, we will discuss possible molecular mechanisms underlying gephyrin postsynaptic clustering, interactions with GABAA receptors, and contribution to GABAergic synapse formation/elimination. According to this view, gephyrin is the core of a dynamic signaling hub that maintains the balance between synaptic excitation and inhibition and therefore contributes to long-term stability of neuronal circuits in the CNS.

Neuroscience I

Commissural GABA release during vestibular compensation in the rat

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The in vivo release of neurotransmitters is commonly measured by microdialysis sampling of the extracellular environment. Microdialysis measurements of biogenic amines correlate well with neurotransmitter release, showing rapidly abolished release after calcium depletion or perfusion with tetrodotoxin. In contrast, microdialysis measurements of GABA indicate slow and incomplete inhibition of release by calcium depletion or tetrodotoxin. The validity of microdialysis measurements for studying amino acid neurotransmitter release is therefore questionable. Amino acid uptake inhibition could increase the synaptic spill-over of neurotransmitter release and thereby increase the validity of microdialysis measurement of amino acid neurotransmitters.

We used microdialysis in alert animals to measure extracellular GABA concentrations in the bilateral medial vestibular nucleus (MVN) at various stages after unilateral labyrinthectomy (UL). UL evokes a prominent behavioural syndrome associated with the silencing of ipsi-lesional neurons and hyperactivity of contra-lesional neurons. The drastic effects of UL on MVN neurons have on indirect grounds been attributed to a large imbalance in the reciprocal commissural inhibitory system that links the bilateral MVNs. When the GABA transporter (GAT1) inhibitor NNC 711 was included in the perfusion solution UL was immediately followed by a difference in GABA concentrations between the two MVNs. In the absence of NNC 711, a change in GABA concentrations could only be observed 24 h after UL.

These findings provide direct evidence that an imbalanced commissural inhibitory system is a root cause of the vestibular deafferentation syndrome, and that GAT1 inhibition can be used to facilitate microdialysis measurements of changes in GABA neurotransmitter release.

The impact of the congenital lack of nNOS on long-term social recognition memory and the olfactory bulb proteome

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The gaseous neurotransmitter nitric oxide synthesizing enzyme neuronal nitric oxide synthase (nNOS) has been suggested to play a major

role in the modulation of memory. We analyzed the short-term and long-term recognition memory of adult nNOS-KO and wild-type mice in an olfactory cues based social discrimination paradigm. Further, a proteomic investigation of the olfactory bulbs of both genotypes were performed both under basal conditions and 6 h after learning, i.e., during the consolidation of long-term memory. Short-term recognition memory was faultless in nNOS-KO mice. However, in contrast to wild-type animals, mutant mice failed to show a long-term olfactory recognition memory. Proteomic analysis revealed changes in glycolytic enzymes (e.g., fructose-bisphosphate aldolase C, glyceraldehyde-3-phosphate dehydrogenase), voltage-dependent anion selective channels 1 and 2, alpha-synuclein, neuronal protein 25/transgelin 3, proteins of the ubiquitin proteasome system, and heterogeneous nuclear ribonucleoproteins implicated in the regulation of messenger RNA trafficking, stability and translation. Our data suggest that in the mouse the congenital absence of nNOS results in an impaired protein synthesis-dependent olfactory long-term memory consolidation within relevant brain structures including the olfactory bulb.

Neuropeptides in stress-related disorders

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A number of neuropeptides seems to be involved in psychiatric disorders, especially corticotropin-releasing hormone (CRH) and vasopressin, which are the main regulators of the hypothalamo-pituitary-adrenal axis (HPA). In affective disorders, the irregularities of the HPA axis are similar to changes during chronic stress, with a more pronounced role of vasopressin. Thus, vasopressin seems to play an important role in the pathophysiology of major depression, while evidence suggests a role for its twin molecule, oxytocin as an endogenous antidepressant/anxiolytic hormone.

The relationship between HPA axis activation and mood disorders with special attention to the role of neuropeptides was in the focus of our research using spontaneously mutated vasopressin deficient Brattleboro rats (di/di).

Behavioral studies confirmed that di/di animals were less anxious in the defensive withdrawal test and revealed lower depressive during forced swimming. The elevated CRH mRNA level in the nucleus paraventricularis hypothalami was unable to compensate the behavioral effect of vasopressin deficiency, while the elevated brain oxytocin levels may contribute to the less anxious/depressive state of vasopressin-deficient animals. Paralelly vasopressin was confirmed to be a prominent regulator of the adrenocorticotrop hormone secretion in wide range of stresses. Accompanied corticosterone elevation was reduced in di/di rats just occasionally and remained elevated longer. Endogenous vasopressin acts as a paracrine signal to facilitate the return of plasma corticosterone to basal levels. Presented data supports the ethiopathogenic role of vasopressin in affective disorders, which seems to be more pronounced than the role of CRH and supported by the opposing changes in oxytocin levels.

In search for ketamine-induced antidepressant-like effects

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Preclinical studies hasten drug discovery, and this concerns also the area of research on antidepressants. Antidepressant-like effects have been demonstrated for a variety of ligands inhibiting glutamatergic neurotransmission, including *N*-methyl-D-aspartic acid (NMDA) receptor antagonists. The effects of ketamine in animals are of particular interest, because this dissociative anesthetic and a noncompetitive NMDA glutamate receptor antagonist produces marked antidepressant effects in depressive patients. Recently, this laboratory replicated findings demonstrating that a single dose of ketamine given immediately before the test reduced immobility in rats and mice. However, no enduring (examined 1–2 weeks later) antidepressant-like effects of a single dose of ketamine was found in the rat forced swim test or a mouse tail-suspension test. We also failed to demonstrate that a single or repeated (2 weeks) ketamine treatment altered apomorphine-induced locomotor activity in rats. Neither acute nor chronic treatment with ketamine affected the density of cortical beta-adrenoceptors. These data suggest that in rodents, ketamine produces neither enduring antidepressant-like effects on immobility after a single administration, nor the antidepressant-like effects (apomorphine challenge, density of cortical beta-adrenoceptors) after chronic treatment. The reasons of why preclinical tests designed to find antidepressant activity are unable to confirm clinical data will be discussed.

Involvement of glutamate and nitric oxide in odour aversion learning

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Animals are able to distinguish between nutritive and noxious food based on chemosensory information, i.e., odour and taste (flavour). Rats readily learn to avoid the consumption of a specific flavour if they experience visceral malaise after its ingestion. While conditioned taste aversion has been extensively studied, the mechanisms underlying conditioned odour aversion (CAO) are much less understood. The glutamate and nitric oxide (NO) neurotransmission systems play a critical role in experience-dependent neuronal plasticity, as well as in a variety of learning tasks. Using intracerebral treatments we explored the role of the NMDA receptor-dependent nitric oxide synthase (NOS) activation into the olfactory bulb (OB) and amygdala (AMY) during acquisition, consolidation and retrieval of COA. Adult male rats implanted with permanent cannulae aimed at OB or AMY were trained in a standard two bottle odour aversion learning paradigm. Different NMDA receptor antagonists (MK-801 and DL-AP4), or NOS inhibitors (L-NAME and 7-NINA) were administered during the different learning phases of COA. Both, NMDA receptors blockade and NOS inhibition into the AMY, dose-dependently disrupted acquisition and

early memory consolidation of COA, without affecting its retrieval; however, only NMDA receptors antagonists impaired COA acquisition, but produced a deeper amnesia when infused into the OB. Thus, while glutamatergic transmission appears to be essential for odour aversion learning, nitrergic mechanisms underlying COA may be site specific.

Neuroscience II

Involvement of NR2B and NR2D subunits in NMDAr-mediated transmission and plasticity in principal cells and interneurons in dentate gyrus

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N-methyl-D-aspartate receptors (NMDARs) have a pivotal role in long-term plasticity and NMDAR-mediated synaptic transmission is subject to plasticity. We have investigated the subunit composition of NMDARs and plasticity of NMDAR-mediated transmission at excitatory synapses on granule cells (GCs) and hilar interneurons (INs) in the dentate gyrus. NMDAR-EPSCs recorded from hilar INs had significantly slower decay kinetics compared to those recorded in GCs (weighted decay time constant $\tau = 31 \pm 1$ ms and 62 ± 5 ms, $n = 25$, for GCs and INs, respectively. Inhibition of NMDAR-EPSCs by the NR2B antagonist ifenprodil (3 μM) was similar in GCs and INs with a depression of ~30%. The NR2D-preferring antagonist UBP141 (3 μM) inhibited NMDAR-EPSCs in INs by ~30% but had no effect on control NMDAR-EPSCs in GCs. High frequency stimulation (HFS) induced LTP of NMDAR-EPSCs in GCs which was postsynaptically expressed via NR2D-containing NMDAR. In contrast, HFS induced a presynaptically expressed LTD of NMDAR-EPSCs in INs but no change in postsynaptic NMDARs.

Neuronal viability is controlled by a functional relation between synaptic and extrasynaptic NMDA receptors

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N-methyl-D-aspartate receptors (NMDARs) are critical for synaptic plasticity but they have also been described as a common source of neuronal damage. However NMDA-R antagonists failed to show neuroprotective efficacy in human clinical trials or produced intolerable adverse effects. The failure of these agents may be attributed to a fundamental misunderstanding of the roles of NMDA-R, particularly their localization in neuronal function and survival. In the present study, we investigate the influence of NMDA-R cellular locations on excitotoxicity and the activation of signalling pathways. By blocking GABAa receptor function cortical neurons fire burst of action potential which resulted in Ca^{2+} plateus visualized by videomicroscopy. This intracellular Ca^{2+} increasing is fully blocked by the co-application of MK801. The exposition of cortical neurons for 24 h to bicuculline, does not induce any neurotoxicity. In order to investigate the role of extrasynaptic NMDA-R, we selectively inactivated synaptic NMDA-R by exposing neurons to MK801 under bicuculline treatment and we performed a bath application of NMDA. In these conditions, we measured an increase in intracellular Ca^{2+} concentration mediated by the activation of extrasynaptic NMDA-R. When applied for 24 h, this condition induced neurotoxicity. In light of the cell death-promoting activities of extrasynaptic NMDA-R, we have determined the pharmacological differences of NMDA-R and characterized the signalling pathways (MAP kinases, CREB) induced by selective activation of synaptic or extrasynaptic NMDA-R. We also demonstrated that the only clinically approved NMDAR antagonist, memantine, preferentially antagonizes extrasynaptic NMDARs.

NR2D-containing NMDA receptors mediate tissue plasminogen activator-promoted neuronal excitotoxicity

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Although the molecular bases of its actions remain debated, tissue-type plasminogen activator (tPA) is a paradoxical brain protease, as it favours some learning/memory processes but increases excitotoxic neuronal death. Here we demonstrate that, in cultured cortical neurons, tPA selectively promotes NR2D-containing *N*-methyl-D-aspartate receptor (NMDAR)-dependent activation. We show the tPA-mediated signalling and neurotoxicity through the NMDAR are blocked by co-application of a NR2D antagonist (PPDA) or knockdown of neuronal NR2D expression. In addition, activation of synaptic NMDAR prevents further tPA-dependent NMDAR-mediated neurotoxicity and sensitivity to PPDA. This study demonstrates that the previously described pro-neurotoxic effect of tPA is mediated by NR2D-containing NMDAR-dependent Erk(½) activation, a deleterious effect prevented by synaptic pre-activation.

Manipulating antioxidant gene expression: endogenous mechanisms and pharmacological approaches

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Antioxidant defences are important for neuronal longevity. These defences can take the form of both intrinsic enzymic systems as well as extrinsic systems, such as those provided by surrounding astrocytes. The regulation of intrinsic and/or extrinsic antioxidant defences may influence the vulnerability of neurons to oxidative insults in vitro or the progression of disorders associated with oxidative stress in vivo. Key intrinsic antioxidant systems in neurons are based around thioredoxin and glutathione. We describe here findings that neuronal activity strongly up-regulates the capacity of both these enzymic systems to detoxify harmful reactive oxygen species such as hydrogen peroxide. The regulation of both systems is associated with changes in gene expression, including the upregulation of sulfiredoxin, glutamate-cysteine ligase and the suppression of thioredoxin-interacting protein. The net effect of these activity-dependent changes is to boost thioredoxin activity, prevent the hyperoxidation of peroxiredoxins, and enhance both rates of glutathione synthesis and peroxidation. We will also discuss ways of mimicking some of these changes to antioxidant gene expression through the use of small molecules, including those which activate gene expression mediated by the antioxidant response element.

NMDA/nitric oxide pathways as targets in psychiatric disorders

Magnesium- and zinc-deficiency models for depression and their relationship to NMDA/NO pathways

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Accumulating evidence suggests that depression may be associated with enhanced *N*-methyl-D-aspartate/nitric oxide (NMDA/NO) signalling. Modelling enhanced NMDA receptor activity in animals is difficult since over-activation can lead to cellular damage and death. As both magnesium and zinc inhibit NMDA receptor activity via modulatory sites, dietary induced magnesium- and zinc-deficiency may be a suitable way to mildly activate NMDA receptors for prolonged periods. Indeed, using validated animal tests of depression, we can show that both magnesium- and zinc-deficient mice display enhanced depression-like behaviour which is sensitive to chronic antidepressant treatments. By quantifying the expression of immediate-early genes (c-Fos/Zif268) we revealed in both mouse models stress-induced hyperactivation of the amygdala, a key region in the processing of emotion and mood in animals and humans. Magnesium-deficiency failed to induce enhanced depression-like behaviour in heterozygous neuronal NO synthase knock out mice, suggesting that activation of NO signalling pathways significantly contributed to the enhanced depression-like behaviour in this animal model. Furthermore, we revealed that magnesium-deficient mice display altered expression of NG,NG-dimethylarginine dimethylaminohydrolase 1, neuronal nitric oxide synthase and NMDA receptor 2B in the amygdala/hypothalamus. These changes were sensitive to chronic antidepressant treatment providing further evidence for an involvement of NMDA/NO signalling. Collectively, these results suggest magnesium- and zinc-deficient mice as new screening models for clinically active antidepressants. Since these models are not based on the monoamine hypothesis of depression, they may be particularly suitable to identify antidepressants with new modes of action, which do not primarily target monoamine systems.

Involvement of NMDA/NO pathway in antidepressant activity of zinc

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Zinc exhibits antidepressant activity in animal screening tests and models of depression. Also the enhancement of antidepressant action of conventional drugs by zinc was demonstrated in preclinical and clinical studies. However, the mechanism of antidepressant activity of zinc is not quite clear. Zinc in a potent antagonist the NMDA receptor and inhibitor of activity of NO synthase. To examine the role of NMDA/NO pathway in zinc antidepressant activity we used the forced swim test (FST). Zinc activity in the FST was antagonized by NMDA and D-serine co-treatment, and on the other hand NMDA antagonists (CGP 37849, L-701,324, D-cycloserine, MK-801) enhanced antidepressant activity of sub-effective dose of zinc. Also, a L-NA (Nitric Oxide Synthase inhibitor) enhanced the antidepressant activity of zinc. Moreover, zinc induced reduction in the glycine potency to inhibit [^{3}H]L-689,560 binding to Gly/NMDA glutamatergic receptors in the rat frontal cortex following chronic treatment. All the data indicate the involvement of NMDA/NO pathway in the antidepressant activity of zinc in animal preclinical measures and suggest such antidepressant mechanism of zinc in human depression.

Stress associated changes in the nitric oxide signaling cascade: relevance for affective psychopathology

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Affective disorders are widely distributed disorders with severe social and economic effects. Several studies suggest that stress is a key mediator in both the precipitation and progression of affective disorders. Fortunately, there is strong evidence underlining that effective treatment helps to restore function and quality of life.

There is accumulating evidence that the novel neurotransmitter nitric oxide (NO) acts as a neuromodulator, participating in several sub-cellular processes underlying cellular memory and neuronal toxicity. As such, nitrergic pathways may have an important role in hippocampal degenerative pathology and cognitive deficits seen in patients with affective disorders. A few clinical and several preclinical studies, strongly suggests involvement of the nitric oxide (NO) signalling pathway in these disorders. Several of the conventional neurotransmitters, including serotonin, glutamate and GABA, are intimately regulated by NO. Moreover, distinct classes of antidepressants (Imipramine, Tianeptine, Citalopram and Paroxetine) have been found to modulate the NO level in the living rat hippocampus in clinically relevant doses, and recent work from our group, using selective inhibitors of phosphodiesterase 5, indicate that the whole NO signalling pathway may play a major role in the behavioural and neurochemical effects observed.

We have also investigated the responses in the nitrergic system following subacute stress in a genetic animal model of depression. Here, our results indicate that key proteins in the *N*-methyl-D-aspartate (NMDA)-NO signalling pathway, including nNOS, PIN and PSD95, were differentially affected following stress in the model of depression, and its' controls.

Taken together, these data further support an important role for the NO cascade in affective disorders.

Mechanisms mediating the psychotropic properties of NO synthase inhibitors

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Nitric oxide (NO) plays an important role in the regulation of behaviour of relevance to psychiatric disorders including aggression, depression and anxiety. Inhibitors of NO synthase (NOS) possess anxiolytic and antidepressant properties in animal models. Mechanisms mediating such properties are less well understood, however inhibition of the neuronal isoform of NOS is most strongly implicated. NO is a key intracellular messenger associated with activation of the glutamate *N*-methyl-D-aspartate receptor (NMDA-R). Since antagonists of NMDA-R possess antidepressant properties, targets downstream of the receptor, such as NOS, may represent targets for antidepressant drug action that lack the problems associated with direct inhibition of NMDA-R. NOS inhibitors dose dependently and stereo-selectively produce antidepressant activity in the forced swimming test, FST, a preclinical behavioural screening procedure sensitive to antidepressant activity. By contrast to NMDA-R antagonists, NOS inhibitors are devoid of locomotor stimulatory properties. The behavioural profile obtained parallels that of selective serotonin re-uptake inhibitors (SSRIs), the most widely prescribed drug treatment for depression and some anxiety disorders. Sub-active doses of NOS inhibitors augment the antidepressant potency of SSRIs. Moreover depletion of endogenous serotonin (5-HT), or inhibition of 5-HT_2 receptors, block the antidepressant-like activity of NOS inhibitors in the FST suggesting that NOS inhibitors elicit their antidepressant-like activity in the FST through a 5-HT dependent mechanism. Our findings have been supported by others and suggest that the psychoactive properties of NOS inhibitors involve glutamate and 5-HT mechanisms that may represent a novel approach for the treatment of depression and anxiety disorders.

Nitric oxide, L-lysine and cognitive dysfunction in schizophrenia: a translational approach

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Cognitive dysfunction is considered a core deficit of schizophrenia and predicts social functioning and long-term outcome in patients. This cognitive dysfunctionality currently lacks effective pharmacological treatment. In order to identify novel drug targets, translational experimental animal models of cognitive dysfunction are required. We have established a behavioural test battery in rodents, ranging from pre-attentive information processing to cognitive flexibility. Using this test battery, we have demonstrated that nitric oxide (NO) synthase inhibitors can ameliorate impairments induced by the schizophrenomimetic drug phencyclidine (PCP), across several levels of cognitive complexity.

In a recent study by our research group L-lysine, a competitive inhibitor of NO synthase substrate, was demonstrated to attenuate the disruptive effect of PCP on pre-attentive information processing. Hence, treatment with L-lysine could have therapeutic effects in patients with schizophrenia. In order to investigate the effects of L-lysine on cognition and symptom severity in patients with schizophrenia, we conducted a proof of concept study. Ten patients received 6 g L-lysine/day as an add-on treatment to their conventional antipsychotic treatment or placebo, in a crossover design, for a total of eight weeks. The blood concentration of L-lysine increased in eight patients after treatment and there were no reports of aversive side effects. Furthermore, the results suggest that there is a beneficial effect of L-lysine treatment on symptom severity, sensory information processing, and cognition. However, a larger study, with higher power, is needed to further explore the potential treatment benefits of L-lysine in patients with schizophrenia.

Oxidative stress of amino acids and proteins

Protein damage and the proteasome

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Protein damage and oxidation is unavoidable consequence of aerobic life. However, a well functioning cellular metabolism is able to recognize and degrade oxidatively damaged proteins. Mammalian cells contain a multitude of proteolytic enzymes characterized by different compartmentalization and substrate specifity. One of the major proteolytic systems in mammalian cells is the proteasomal system. Besides the 20S core proteasome it consists of several components and is strongly redox-regulated. In living mammalian cells it was demonstrated that the proteasome is the major proteolytic system responsible for the degradation of oxidized proteins and that the exposure of cells to oxidants is followed by an enhanced protein turnover. This turnover is directed towards the selective removal of oxidatively modified proteins by the proteasomal system. We demonstrated that the isolated proteasome is able to degrade moderately oxidized proteins, whereas severely oxidized model proteins are poor substrates of this protease. Several lines of evidence demonstrate that the recognition of oxidized proteins by the protease is due to unfolding and to the exposure of hydrophobic moieties on the protein surface.

A failure of an adequate degradation of oxidized proteins is leading to various side effects, including formation of protein aggregates. Such protein aggregates are major contributors to several pathologies. Therefore, it is increasingly recognized that malfunction of the proteasomal system is playing a major role in several diseases (including neurodegenerative diseases) and the aging process.

Enabling the quantitation of post translational modifications

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Identifying post translational modifications (PTMs) is essential to understanding protein function and activity. Mass spectrometry can identify expected PTMs but the number of known possible PTMs is too large to analyze a dataset for all of them. It is, however, possible to test a small number of abundant PTMs such as phosphorylation.

For understanding changes in function and activity under different physiological conditions it is not only necessary to identify PTMs they

also need to be quantified and put into their experimental context. We earlier developed 2DB, a proteomics database able to store such information. An extension to 2DB enabling different modes of quantitation has also been developed recently. This tool has been extended to map not only identified peptides to their experimental context but also their PTMs. The relative amount of PTMs per peptide, protein, and fraction of a separation can be deduced using this tool. Furthermore, between different experiments peptides and proteins could be quantified with the previous tool. The additions make the results more sound since PTMs are now included into the analysis. The PTMs not only increase the potential for more spectral counts and thus a better quantification of peptides and proteins but can be quantified in their own right. Between several experiments the overall difference in identified PTMs can be calculated as well as the differential modification of peptides and proteins, overall, or in specific fractions or pools thereof.

Post-translational actin nitration: targets and functional consequences

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To link the phenomena of inflammatory-induced increases in protein nitrotyrosine (NO2Tyr) derivatives to protein dysfunction and consequent pathological conditions, evaluation of discrete NO2Tyr modifications on specific proteins must be undertaken. Actin, one of the most abundant proteins in eukaryotic cells, constitutes 5% or more of cell protein and serves with other cytoskeletal proteins such as tubulin as a critical target for nitration-induced functional impairment. Mass spectrometric (MS) proteomics-based strategies allow for the identification of all individual proteins that are nitrated by separating tissue homogenates using 2D gel electrophoresis, detecting the nitrated proteins using an anti-NO2Tyr antibody, and then identifying the peptides generated during an in-gel proteolytic digest using matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) MS. In order to fully understand the functional alterations induced by NO2Tyr, identification of specific sites of NO2Tyr modifications provides powerful insight into the implications of protein nitration. To more incisively detect biological NO2Tyr levels, isotope dilution gas chromatography (GC) joined with mass spectrometry (MS) detection methods on chemically hydrolyzed tissue and fluid samples are employed. Typically, after the addition of isotope-labeled internal standards to a delipidated sample, the protein is hydrolyzed and the resulting amino acids are isolated from the hydrolysate by chromatography on a solid phase extraction column prior to derivatization. While sometimes technically challenging, the analytical MS techniques evaluating NO2Tyr-containing proteins to date may provide the most accurate information regarding the location and magnitude of NO2Tyr formation in vivo, especially when used in conjunction with immunohistochemical approaches.

Redox signalling and detection of protein oxidation by mass spectrometry

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It is now recognised that redox control of proteins plays an important role in many signalling pathways both in health and disease. Proteins can undergo a wide variety of oxidative post-translational modifications (oxPTM); while the reversible modifications are thought to be most important in physiological processes, non-reversible oxPTM may contribute to pathological situations and disease. The oxidant is also important in determining the type of oxPTM (chlorination, nitration, etc.), and the susceptibilities of residues vary depending on their structural location. The best characterized oxPTMs involved in signalling modulation are partial oxidations of cysteine to the disulfide, glutathionylated or sulfenic acid forms, but there is increasing evidence that specific oxidations of methionine and tyrosine may have some biological roles. Well understood examples of oxidative regulation include protein tyrosine phosphatases, e.g. PTP1B/C, and members of the MAPK pathways such as MEKK1 and ASK1. Transcription factors such as NFkB and Nrf-2 are also regulated by redox-active cysteines. Improved methods for analysing specific oxPTMs in biological samples are critical for understanding the physiological and pathological roles of these changes, and tandem or MS^3 mass spectrometry techniques interfaced with nano-LC separation are being now used. MS^3 fragmentation markers for a variety of oxidized residues including tyrosine, tryptophan and proline have been identified, and a precursor ion scanning method that allows the selective identification of these oxPTMs in complex samples has been developed. Such advances in technology offer potential for biomarker development, disease diagnosis and understanding pathology.

Relation between activities of antioxidant enzymes and clod tolerance of postharvest tomato fruits from two cultivars at cold storage

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In order to find the relation between postharvest fruits from cultivars with different chilling sensitivity and antioxidant enzyme activities, postharvest tomato fruits from two cultivars, Santiam (*Solanum lycopersicum* L. cv. Santiam) and Lichun (*Solanum lycopersicum* L. cv. Lichun), differing in chilling tolerance, were treated with short-time (24 h) and long-time (20 days) chilling stress. Activities of antioxidant enzymes, catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD) and superoxide dismutase (SOD) were detected. It was found that enzyme activities reflected chilling tolerance of cultivars, and showed a positive relationship between them. Activities of CAT, POD and SOD could swiftly reflect chilling tolerance diversity between the two cultivars within 4–24 h under chilling stress in a significant level ($P < 0.05$). Conclusively, it was suggested that detecting activities of antioxidant enzymes was a fast and effective method to judge chilling tolerance of postharvest fruits.

Purification, characterization and effect of antioxidative dipeptide from salmon protamine hydrolysate: study on the properties of bioactive peptides

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Protamine, which was obtained from fish milt that is normally discarded as an industrial by-product in the process of fish plant, was

hydrolyzed with trypsin, etc. We found salmon protamine hydrolysate possessed antioxidant activity in vitro and in vivo. To investigate characterization of anti-oxidative peptides, protamine hydrolysate was further purified by the chromatography according to the oxidation systems. The supernatants were applied onto a Sephadex G-35 column. The fraction showing highest antioxidative activities was further chromatographied on a manually prepared Macro-Prep High Q column, Hi-Trap desalting column, an YMC-Pack Protein-RP column and Symmetry Shield RP18 column. The molecular mass and the amino acid sequence of the purified peptide were identified by LC–MS data and the database search in NCBI. The most potent peptide derived by successive chromatography isolation was identified to be Pro-Arg matching 1–2 and 16–17 residues of the salmon protamine.

In this future study, we compared the antioxidant capabilities of purified and synthesized dipeptide (Pro-Arg) and found both of them had very strong hydroxyl-radical scavenging activity in vitro, even at a very low concentration. The protective effect of the dipeptide against oxidative stress was evaluated using H_2O_2-induced oxidative stress of human diploid fibroblasts MRC-5 cell model. These results showed that dipeptide pretreatment could eliminate about 90% intracellular ROS induced by H_2O_2 when the concentration of synthesized dipeptide reached 250 μg/ml. Meanwhile, the dipeptide normalized S phase arrest of MRC-5 cells exposed to H_2O_2. Our findings demonstrate that Pro-Arg can protect against oxidative stress/damage and H_2O_2-induced human diploid fibroblasts cell death.

Analysis of oxidative folding of ribonuclease A by using water-soluble selenoxide reagent

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The SS bonds in a protein molecule are important for maintaining the native 3D structure. In our laboratory, we have recently succeeded in observation of slow SS rearrangement reactions separately from fast SS formation reactions in the oxidative refolding pathways of ribonuclease A (RNase A) by using 3,4-dihydroxyselenolane oxide (DHS) as a highly efficient and quantitative oxidizing reagent. Thus, facile observation of the partially oxidized key intermediates was achieved. Herein, we investigated on the temperature effects to obtain information about thermodynamic stabilities and structures of the key intermediates.

Native RNase A (N) was reduced with DTT to the reduced form (R). The folding reaction was initiated by addition of DHS under the conditions at 5–45 degrees Celsius and pH 8.0 and stopped by addition of AEMTS, a SH blocking reagent, with variation of the reaction time. After desalting by gel filtration chromatography, the obtained refolding intermediates were analyzed by HPLC.

It was revealed that the oxidation reaction (i.e., SS formation) was completed quantitatively within 1 minute in the applied temperature range and then N and the four key folding intermediates (des intermediates) were gradually generated by slow SS rearrangement reactions. The relative stabilities of the des intermediates were found to be sensitive to the temperature. At low temperatures, des[40–95] was thermodynamically most stable, whereas the heat denaturation temperature was highest for des[65–72] among the four des intermediates.

Oxidative interactions of aminothiols in buffer versus physiological fluid

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The aminothiols cysteine (Cys) and homocysteine (Hcy) were incubated separately and together in minimal medium (a buffered salt solution) as a preliminary to the investigation of their possible stabilising affect on the oxidation of glutathione under the influence of cultured astrocytes.

In the absence of copper, Cu(II), Cys was stable over 4 h at 370°C whilst Hcy oxidised slowly. Surprisingly, when co-incubated both were stable to oxidation. Incubated separately in the presence of Cu(II) both oxidised very rapidly to form the corresponding disulphide and a mixture of sulphinic and sulphonic acids. This was not altered when co-incubated; however the oxidation products again included Cys/Hcy sulphinic and sulphonic acids but, additionally, the mixed disulphide formed also degraded rapidly and contributed to the formation of these compounds.

This was repeated in CSF as a (virtually) protein free alternative to plasma and the above spurious affects were notably absent.

Conclusions:

1. In the absence of copper, thiols may exhibit a mutually protective affect.
2. All thiol species need to be measured or spurious products may go unnoticed.
3. These affects are not present in physiological fluid (CSF).
4. Oxidation experiments performed in aqueous media may not adequately represent that which may occur physiological fluid.

Comparison of electrochemical and spectrometric detection of-SH moieties

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Low molecular mass thiols such as glutathione play key roles in heavy metals metabolism and scavenging of free radicals. Free –SH moieties of the above-mentioned compounds are highly reactive and are often found conjugated to other molecules. Searching of suitable instruments and procedures for detection of free –SH moieties presents important analytical task for decades. Due its reactivity, free –SH moiety can be detected by several types of methods including spectrometry and/or electrochemistry. In this study, we focused on

detection and quantification of –SH moieties by using of electrochemical and spectrometric detection. To calibrate both methods we used *N*-acetylcysteine in micromolar concentrations. The electrochemical method was based on detection of signals caused by proteins containing –SH groups in the presence of cobalt(III) ions. One of these signals called Cat2 was proportional to concentration of –SH moieties. Relative standard deviation was 4% and detection limit was estimated down to submicromolar. Besides electrochemistry, spectrometric analysis was performed at 420 nm (reaction of thiol with Ellman's reagent). The results obtained by both methods correlated very well. Moreover, –SH groups in cysteine, homocysteine, glutathione and metallothionein were detected by the mentioned methods. The proposed methods were subsequently used to detect thiols in fish exposed to stress caused by heavy metals ions (silver or cisplatin). The contents determined by electrochemical and spectrometric methods were correlated. Contents of free –SH groups were also determined after precipitation (12% trichloroacetic acid).

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Adsorptive transfer stripping techniques coupled with voltammetry as a tool for detection of sulphur-containing aminoacids in nanolitres

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Electrochemical methods using hanging mercury drop electrode belong to the most sensitive one for detection of thiols. We report on the improvement of adsorptive transfer stripping technique (AdTS) coupled with differential pulse voltammetry Brdicka reaction. The current technique has been unable to generate reproducible results when analyzing very low sample volumes (500 nL–3 μL). This obstacle can be overcome technically by modifying the current transfer technique including cooling step (4°C) of the adsorbed analyte. The cooling enabled us to prolong time of accumulation (hundreds of seconds) without evaporation of low volumes of a sample used in our experiments. We tested the technique on determination of human serum albumin and promising tumour disease marker protein metallothionein (MT). We determined signals called Cat1, Cat2 and Cat3 corresponding to hydrogen evolution from supporting electrolyte and protein-complex with Brdicka solution (RS_2Co). The observed signals were well repeatable and developed. In addition, we measured the calibration dependences within the interval from 10 pM to 500 nM. The detection limit (3 S/N) of MT or has estimated as 3 signal/noise were down to units of pM. Further the improved AdTS technique was utilized to analyze blood serum samples from patients with cancer. Based on the results obtained it can be concluded that the improved technique can be used to detect a thiol-protein in very low sample volumes and can also prevent interferences during the washing and transferring step.

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DPPH radical scavenging effect of *N*-phenylpropenoyl amides of some biogenic monoamines

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Hydroxycinnamic acid amides are a widely distributed group of plant secondary metabolites. Their biosynthesis and subsequent polymerization in the plant cell wall are generally accepted as integral components of plant defense responses to pathogen challenge, wounding, heavy metals, etc.

Sixteen amides of cinnamic, ferulic, sinapic and *p*-coumaric acids with biogenic monoamines (tyramine, tryptamine, dopamine and 2-phenylethylamine) were synthesized. Their structure was confirmed by UV, ^{1}H-NMR and MS.

The antioxidative potential of the synthesized amides was studied against DPPH* (1,1-diphenyl-2-picrylhydrazyl radical) in abs. EtOH and compared with those of hydroxycinnamoyl amino acid amides and well-known antioxidants.

Synthesis of esters of hydroxycinnamic acids with N-protected amino alcohols and evaluation of their radical scavenging activity

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From the literature it is known that esters of hydroxycinnamic acid with serine, homoserine and tyrosine exhibit strong radical scavenging activity.

In this study, feruloyl- and sinapoyl- esters of N-protected amino alcohols have been synthesized. The N-protected amino alcohols have been obtained by chemoselective reduction of the corresponding mixed anhydride of N-protected amino acids using $NaBH_4$. The newly synthesized esters have been characterized by UV, ^{1}H-NMR and EI-MS.

The study of radical scavenging activity of the synthesized esters is in progress.

Oxidative stress, reactive species and antioxidants Repair of protein radicals

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Hydroxyl radicals and peroxynitrous acid oxidize proteins, which ultimately results in their denaturation. Random radical generation on

the surface of the protein without dioxygen present leads to intramolecular electron transfer, with the last step the oxidation of tyrosine by the tryptophanyl radical. In the presence of dioxygen aliphatic radicals form oxidizing peroxyl radicals. Once a radical is formed, can it be repaired, does it react with dioxygen or is it "repaired" by intramolecular electron transfer?

Glutathione repairs a tryptophan radical in lysozyme with a rate constant of $(1.05 \pm 0.05) \times 105\ M^{-1}\ s^{-1}$, while ascorbate repairs tryptophanyl and tyrosyl radicals ca. three orders of magnitude faster. Glutathione generally reacts slowly, such that formation of peroxyl radicals cannot be prevented. These peroxyl radicals are reduced by glutathione to hydroperoxides, a process that cannot be characterized as a repair. Furthermore, the resulting thiyl radical is capable of hydrogen abstraction. Although physiologically not significant, seleno-glutathione reduces tyrosyl radicals as fast as ascorbate. The reaction of protein radicals (insulin, β-lactoglobulin, pepsin, chymotrypsin, and bovine serum albumin) with ascorbate is competitive with dioxygen, leaves an innocuous ascorbyl radical, and is therefore a true repair. The well-documented loss of ascorbate in living organisms subjected to oxidative stress may result from repair of protein radicals.

A catalytic role for cysteine thiyl radicals in oxidative protein damage

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Redox modification represents a major degradation pathway for proteins in vivo and in vitro. The parameters controlling protein oxidation are not as well understood as it would be desirable to rationally predict the sensitivity of proteins towards oxidation. An important feature of the reaction of a protein with a reactive oxygen species/free radical is that the initial site of attack may not necessarily represent the final location of an oxidation site. In this presentation, we will demonstrate the potential of Cys residues to serve as a point of primary attack with intermediary Cys thiyl radicals subsequently attacking other amino acids within the protein sequence. Thiyl radicals can either involve in reversible hydrogen transfer reactions, electron transfer, or in addition reactions to aromatic amino acid residues. The yields of these reactions depend on primary and secondary structure. An important feature of the hydrogen transfer reactions is the formation of carbon-centered radicals at the peptide backbone. The latter may serve as origin for the formation of peroxyl radicals. In addition, reverse hydrogen transfer from the thiol may lead to the formation of D-amino acids, and examples for such reactions will be provided.

Why do proteins use selenocysteine instead of cysteine?

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Thiyl radicals abstract hydrogen from a protein backbone within microseconds, whereas selanyl radicals cannot. Carbon centered radicals produced by hydrogen abstraction at the protein backbone react with oxygen to form peroxyl radicals and thus initiate a broad range of unwanted reactions.

Selenocysteine catalyses the formation of disulfide bonds from thiols and vice versa. This fact has been used as reason for the biological use of selenocysteine. However, a thioredoxin reductase equipped with a GCUG active site has the same activity as one with SCCS. The expensive incorporation of selenocysteine does not improve its primary function, the catalysis of two-electron transfers. However, if one-electron transfers occur, selenium would protect from the formation of thiyl radicals and its deleterious consequences—thereby increase the lifespan of the protein and prevent the formation of protein hydroperoxides.

After one electron reduction of (RSe-SR′) to $(RSe^{\therefore}SR')^{\bullet}$ the resulting three electron bonded species will immediately dissociate to a thiol and a $R'Se^{\bullet}$ radical. In the sulfur-only analog, approximately 30% of the species are dissociated in equilibrium. Nevertheless, the high reactivity of the thiyl radicals is expected to result in considerable damage to the protein.

Manganese superoxide dismutase: beyond life and death

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Manganese superoxide dismutase (MnSOD) is a nuclear-encoded antioxidant enzyme that localizes to the mitochondria. Expression of MnSOD is essential for the survival of aerobic life. Transgenic mice expressing luciferase reporter gene under the control of the human MnSOD promoter demonstrate that the level of MnSOD is reduced prior to the formation of cancer. Overexpression of MnSOD in transgenic mice reduces the incidences and multiplicity of papillomas in the DMBA/TPA skin carcinogenesis model. However, MnSOD deficiency does not lead to an enhanced tumorigenicity of skin tissue similarly treated because MnSOD can modulate both the p53 mediated apoptosis and AP-1 mediated cell proliferation pathways. Apoptosis is associated with an increase in mitochondrial levels of p53 suggesting a link between MnSOD deficiency and mitochondrial-mediated apoptosis. Activation of p53 is preventable by application of a SOD mimetic ($MnTE\text{-}2\text{-}PyP^{5+}$). Thus, p53 translocation to mitochondria and subsequent inactivation of MnSOD explain the observed mitochondrial dysfunction that leads to transcription-dependent mechanisms of p53-induced apoptosis. Administration of $MnTE\text{-}2\text{-}PyP^{5+}$ following apoptosis but prior to proliferation leads to suppression of protein carbonyls and reduces the activity of AP-1 and the level of proliferating cellular nuclear antigen, without reducing the activity of p53 or DNA fragmentation following TPA treatment. Remarkably, the incidence and multiplicity of skin tumors are drastically reduced in mice that received $MnTE\text{-}2\text{-}PyP^{5+}$ prior to cell proliferation. The results demonstrate the role of MnSOD beyond its essential role for survival and suggest a novel strategy for an antioxidant approach to cancer intervention.

Free radical control of signal transduction pathways during cancer therapy

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Free radicals play key roles in cancer therapy. Many cancer treatment agents, including radiation therapy and a variety of

chemotherapeutic agents, inflict damage to cancer cells through generation free radicals. Our group has investigated the roles of free radicals in tumor response to radiation therapy. Specifically, we have studies how tumors activate various signal transduction pathways to counter the tumoricidal effects of ionizing radiation. We have focused on several known pathways, i.e., the hypoxia-inducible factors (HIF), the epidermal growth factor response pathways, and the apoptotic pathways. In each of these pathways, we have designed novel molecular imaging reporter systems to monitor the activities of the pathway in vivo. Our reporters are based on the firefly luciferase which allows for non-invasive imaging of tumor cells in mice, which greatly facilitates the study the signal transduction pathways in vivo. Using these molecular imaging reporters, we have been able to investigate the roles of reactive oxygen and nitrogen species in tumor response to radiotherapy in vivo. In one example, we have identified a key role for nitric oxide in mediating the stabilization of HIF-1α in tumor cells. This stabilization is hypoxia-independent and mediated through the *S*-nitrosylation of a cysteine residue in the HIF-1α protein. The stabilized HIF-1a mediated increased VEGF secretion and vascular survival, which plays key roles in tumor repopulation after radiotherapy. In summary, the use of our molecular imaging promoters has allowed us to gain significant mechanistic insights into the molecular pathways involved in tumor response to cytotoxic therapy.

Targeting nitroxidative stress in acute and chronic pain

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Pain is a significant global health problem. The economic impact of pain is equally large at approximately $100 billion annually in the USA alone. While selective cyclooxygenase-2 inhibitors are effective for several forms of chronic pain, their occasional side-effects including increased risks of heart attack and stroke prompted the precipitous withdrawal of some of them (i.e. Vioxx) from the market in 2004. Morphine sulfate and other opiate/narcotic analgesics are the most effective treatments for acute and chronic severe pain. However, their clinical utility is often hampered by the development of analgesic tolerance which necessitates escalating doses to achieve equivalent pain relief. This complex pathophysiological cycle contributes significantly to decreased quality of life in the growing population of subjects with chronic pain due to oversedation, reduced physical activity, respiratory depression, constipation, potential for addiction, and other side-effects. Considerable evidence implicates nitroxidative stress in the development of pain of several etiologies and importantly in opiate antinociceptive tolerance, caused by the presence of superoxide, nitric oxide and more recently peroxynitrite that is the product of their interaction. I will discuss the importance of nitroxidative stress in acute and chronic pain and will argue that peroxynitrite is a rational target for therapeutic intervention in pain management. These concepts provide a pharmacological basis for developing inhibitors of peroxynitrite biosynthesis as novel non-narcotic analgesics, thus addressing a large and currently unmet medical need with major socioeconomic consequences.

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Mn porphyrins suppress oxidative stress injuries through redox-based pathways

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Positively charged Mn(III)*N*-alkylpyridylporphyrins (MnP) possess electrostatic and thermodynamic facilitation for the reaction with superoxide ($O_2^{\bullet-}$) and peroxynitrite ($ONOO^-$); some members of the series are among most potent catalytic scavengers of those reactive species with $\log k_{cat}$ ($O_2^{\bullet-}$) equaling $\log k_{cat}$ for the SOD enzymes and with $k_{red}(ONOO^-) > 10^7\ M^{-1}\ s^{-1}$. Reactive species (RS) are widely viewed as signaling molecules controlling cellular transcriptional activity. Thus, by scavenging RS, MnPs prevent activation of redox-active transcription factors AP-1, NF-kB, and HIF-1α, and in turn attenuate oxidative stress in animal models of cancer, central nervous system (CNS) disorders, radiation, diabetes, chronic morphine tolerance, ischemia/reperfusion, etc. MnPs suppress radiation damage even when given 8 weeks after radiation due to the suppression of HIF-mediated secondary inflammatory pathways. One-week intracerebroventricular administration of MnTDE-2-ImP, starting 90-min post middle-cerebral-artery occlusion, caused reduction in cerebral infarct size and neurologic deficit at 8 weeks post-ischemia, due to reduction of primary oxidative stress and suppression of NF-kB-mediated secondary inflammation. Factors other than redox-based antioxidant potency, primarily lipophilicity, also contribute to *in vivo* efficacy. MnTnHex-2-PyP possesses same antioxidant potency as MnTE-2-PyP and MnTDE-2-ImP but is 13,800- and 2,000-fold more lipophilic, accumulates more in cells and is up to 120-fold more efficacious in vivo. MnTnHex-2-PyP is superior where transport across lipid membranes is critical, i.e. in CNS injuries. MnTnHex-2-PyP, but not MnTE-2-PyP, showed remarkable efficacy in rabbit model of cerebral palsy, and is potent in stroke model even when given intravenously at 6 h post-ischemia (0.225 mg/kg), followed by one week subcutaneous injections.

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Zn(II) *N*-alkylpyridyl-porphyrin photosensitizers target structural proteins and enzymes: Impact of lipophilicity

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It is generally accepted that the main cell-damaging factor in photodynamic therapy (PDT) is singlet oxygen. Because of its short life, $^1O_2$ damages targets in close proximity of its site of formation, which in turn is determined by the cellular localization of the photosensitizer. Porphyrins offer almost limitless possibilities for modifications, which can affect cellular uptake and distribution of the photosensitizer. Aliphatic substitutions in the periphery of the porphyrin ring, modulating the hydrophobicity of the molecule, will favor its partition into membrane lipid bilayers or in the hydrophilic regions of the cell. Thus, by proper modifications, porphyrin-based photosensitizers, and as consequence PDT-induced call damage, can be directed to selected cellular compartments. We

have found that upon illumination with visible light, Zn(II) *N*-alkylpyridyl-porphyrins (ZnP) kill bacteria and tumor cells with efficiency exceeding that of a classical photosensitizer, hematoporphyrin derivative (HpD). Illumination of cells loaded with hydrophilic ZnPs inactivate glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, $NADP^+$-linked isocitrate dehydrogenase, aconitase, and fumarase. ZnPs also cause photo-dependent crosslinking of membrane proteins, and irreversible membrane damage. Increases in the hydrophobicity of the ZnP molecule, by attachment of longer aliphatic chains to the porphyrin periphery, increased ZnP accumulation in cell membranes and augmented photosensitizer efficiency by one order of magnitude.

Peptide probes for molecular imaging

Molecular imaging of malignant melanoma with novel PET probes

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Malignant melanoma is one of the most lethal cancers, and it represents a significant public health problem in Europe and the United States. Early diagnosis and accurate staging of melanoma is crucial for improvement of the survival of melanoma patients. Positron emission tomography (PET) is a promising technology for non-invasively imaging micrometastases. PET coupled with a proper imaging probe may provide oncologists a highly sensitive procedure for early detection of melanoma and accurate staging of high-risk melanoma patients. A variety of PET probes have been developed for imaging different melanoma associated molecular targets in our laboratories. These targets include melanin, melanocotin receptor 1 (MC1R), and integrin $\alpha_v\beta_3$. The application of these PET probes for melanoma imaging will be reviewed and discussed in this presentation.

Phage display-derived ErbB-2-targeting peptides for cancer imaging

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Small peptides are effective vehicles for targeted delivery of radionuclides for imaging and therapy due to their ability to bind specific targets on human cancers. The ErbB-2 receptor contributes to the invasion and progression of several cancers including those of the breast, prostate and ovary. In this study we investigated the ability of a hexameric peptide, KCCYSL, selected through bacteriophage (phage) display against the ErbB-2 receptor, to bind breast and ovarian carcinoma cells. The bifunctional-chelator DOTA was attached to the KCCYSL peptide and radiolabeled with ^{111}In for single photon emission computed tomography (SPECT/CT) imaging, and the chelators CB-TE2A and NOTA were coupled to the peptide for labeling with ^{64}Cu for positron emission computed tomography/CT imaging. *In vitro* breast and ovarian carcinoma cell binding studies were performed. In vivo biodistribution and imaging studies with the radiolabeled peptides were evaluated in human breast and ovarian tumor xenografted SCID mice.

The radiolabeled peptides bound to human MDA-MB-435 breast and Ovcar-3 ovarian carcinoma cells with high specificity as determined by the competition studies. Evaluation of cell binding data indicated that the radiolabeled peptides exhibited IC_{50}'s of $\sim$50.0 nM. In vivo biodistribution studies demonstrated radiolabeled peptide accumulation in tumors. Little or no accumulation occurred in other organs except the kidney and liver and depended on the chelate and radionuclide utilized. Imaging studies showed good tumor uptake for all peptides after 1–2 h post-injection. Thus, the phage display derived peptide once radiolabeled with ^{111}In or ^{64}Cu, can function as a non-invasive in vivo imaging agent of human ErbB-2-expressing tumors.

Direct ^{18}F-labeling of peptides for imaging gastrin releasing peptide receptor expression

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Positron emission tomography allows the visualization of human physiology by detection of short-lived positron emitting targeted radiopharmaceuticals. It is the only non-invasive technology that can routinely and quantitatively measure metabolic, biochemical and functional activity in living tissue.

Due to their favourable pharmacokinetics, peptides are attractive tools for the visualization and assessment of pathological tissue. Even though several methods for the attachment of a positron emitting nuclide to peptides have been developed, commercialization of such tracers is hampered by insufficient labelling yields, low specific activities, or time consuming multi-step labelling methods.

The aim of the presented research is the development of reliable, high affinity one-step labelling of peptides using F-18 potassium fluoride. Based on the reactivity of the fluoride ion, different methods for the direct fluorination were evaluated and successfully used for the labelling of a GRP receptor targeting peptide. The resulting fluorine-18 labelled lead peptide shows high tumor uptake in PC-3 and LnCaP xenograft mice models.

Tissue-specific homing peptides for molecular imaging of apoptosis

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Apoptosis plays an important role in the pathogenesis of a variety of diseases, such as cancer, myocardial and cerebral ischemia, and neurodegenerative diseases. Imaging apoptosis, therefore, may be essential for the management of these diseases. Annexin V, a protein that binds to phosphatidylserine on apoptotic cell surface, is currently the most popular probe for imaging apoptosis. Small peptides might be a potential alternative to bulky proteins or

antibodies for diagnostic applications. They often have better tissue penetration and less possibility of immunogenicity. Using phage display technology, we have identified a peptide probe that targets apoptotic cells and named it ApoPep-1 (apoptosis-targeting peptide-1). When injected intravenously into nude mice bearing a tumor xenograft, fluorescein-conjugated ApoPep-1 selectively homed to apoptotic at tumor tissues, which was demonstrated by TUNEL and caspase staining. Moreover, ApoPep-1 bound to etoposide-induced apoptotic tumor cells on culture at higher levels than that to non-apoptotic cells. In vivo imaging of mice after systemic administration of Cy7.5-conjugated ApoPep-1 demonstrated a strong near-infrared fluorescence signal at tumors of doxorubicin-treated mice as compared to untreated control mice. Little fluorescence signal was observed at tumors of doxorubicin-treated mice injected with a control peptide. These results suggest that ApoPep-1 is a promising probe for imaging apoptosis of tumor and monitoring tumor response to therapy.

Landscape phage probes for human cancer cells

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Rapid identification of probes that recognize specific cells in living body would have far-reaching applications in medical science for identification of specific molecular targets, in vivo imaging, and navigating cytotoxic medicines to a site of disease. The concept of using phage display libraries for selection of targeting ligands has been proved for the past decade both in cell culture, model animal and clinical human patient settings. The selected phage-derived probes can be used for development of selective, stable, active and physiologically acceptable ligands that would navigate the imaging label and anticancer therapeutics to the tumor site. Here we describe a new source of bioselective imaging probes with enhanced performance—landscape phage—substitute antibodies with high selectivity, affinity and stability. The phage borne breast, prostate and glioma tumor-specific peptides genetically fused to the phage major coat protein have been affinity selected by their ability to specifically bind cancer cells, and/or penetrate into the cells. The selected tumor-specific phage can be converted into imaging probes by their conjugation with imaging labels. Thus, the major principle of the phage probe concept is that targeted phage-derived molecular probes recognize the same receptors, cells, tissues and organs that have been used for selection of the precisely targeting landscape phage. Using bioselected phage particles—naturally assembled structurally perfect multivalent peptide dendrimers, as diagnostic probes can provide much higher selectivity and sensitivity of analysis in comparison with individually synthesized peptides.

Molecular imaging of the receptor for advanced glycation endproducts

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The receptor for advanced glycation endproducts (RAGE) is a member of the immunoglobulin superfamily and has been implicated in the pathogenesis of various disorders including inflammatory processes and cancerogenesis. However, data concerning the functional expression of RAGE in inflammatory compartments and other pathologies in vivo are scarce. We report a multi-radiotracer approach using radiolabeling of various RAGE ligands, including glycated low-density lipoproteins (glycLDL), glycated albumin (glycBSA), and S100 proteins (S100B and S100A12) with the positron emitter fluorine-18 (^{18}F) and the application of ^{18}F-labeled RAGE ligands in dynamic small animal positron emission tomography (PET) studies. Radiolabeling of proteins was performed by conjugation with *N*-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) causing no adverse alterations of the biological functionality of the proteins in vitro. Biodistribution and metabolite studies in rodent normal, inflammatory, and tumor models revealed high stability for the ^{18}F-RAGE ligands in vivo. The in vivo kinetics of ^{18}F-RAGE ligands, with or without presence of specific ligands or inhibitors of RAGE and, additionally, various scavenger receptors, in rodent models was quantified by PET, and correlated well with the anatomical localization of RAGE, e.g., in lung, endothelium, inflammatory lesions, and tumors. ^{18}F-radiolabeling of glycLDL, glycBSA, and S100 proteins and the use of small animal PET provide a potential approach to measure the functional expression of RAGE under normal and pathophysiological conditions in vivo.

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Peptide use for drug delivery and imaging

Cell-penetrating peptides in design, synthesis and applications of oligonucleotide delivery

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Recent achievements and our recent data are summarized on improved drug delivery by cell-penetrating peptides on the variety of different cargoes including: DNA, PNA, mRNA, peptides and proteins. These cargoes are applied to regulate gene expression in live cells as decoy dsDNA, antisense and siRNA oligonucleotides. The peptides are used as protein mimics participating in gene expression. We also report on tumour cell targeting by application of CPP in vivo; this technology holds great promise in further drug development for tumour treatment.

Application of peptides in diagnostic imaging

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The discovery that certain tumour types over-express receptors for peptide hormones dates back to the mid 1980s. For example, the high level of expression of somatostatin receptors on various tumours has provided the molecular basis for the successful use of radiolabelled somatostatin analogues (^{111}In-DTPA-[D-Phe1]-octreotide) as tumour tracers in nuclear medicine. Since then, there has been a growth in the development of radiolabelled peptides for diagnostic applications, primarily driven by the fact that peptides

have the ideal characteristics for nuclear imaging: reproducible radiolabelling, fast in vivo clearance, rapid tissue penetration and low antigenicity.

For evaluation of tumour receptor expression, different radiolabelled peptide analogues such as somatostatin, cholecystokinin, gastrin, bombesin, substance P, vasoactive intestinal peptide, neuropeptide-Y and glucagon-like peptide-1 analogues have all been developed and most recently, peptides targeted to integrins such as the $\alpha v\beta 3/\beta 5$ integrin, over-expressed during the process of tumour growth.

Within GE Healthcare we have developed an ^{18}F-radiolabeled small peptide (AH111585) containing an RGD (Arg-Gly-Asp) sequence which binds with high affinity to $\alpha v\beta 3/\beta 5$ integrin, a cell adhesion receptor that is highly expressed on tumor neovasculature, but expressed at low levels on mature endothelium and epithelial cells. We have demonstrated specific tumour uptake of this radiolabelled peptide both preclinically and clinically. In addition, [^{18}F]AH111585 has also been used to examine the response of human tumor xenografts to treatment with novel anti-angiogenic therapies.

In summary, radiolabelled peptides continue to offer new horizons for diagnostic imaging, and will enhance our understanding of how novel therapeutic strategies impact upon tumours.

Cell penetrating peptides: design, cellular trafficking and applications to the delivery of oligonucleotides

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Improved delivery strategies for biomolecules as peptides or nucleic acids have long been searched for since poor translocation across membrane barriers is a major limitation for many of their clinical applications. While efficient for the delivery of conjugated peptides or of fused proteins, cell penetrating peptides (CPP) as Tat48-60, Penetratin or oligoArg have turned out to be poorly efficient for the transfection of steric-block oligonucleotides (ON).

We have designed Arg-rich CPPs such as $(RAhxR)_n$ (in which Arg residues are interspersed with 4-aminohexanoic acid) or Pip (a series of Arg-extended Penetratin derivatives engineered for improved metabolic stability) which are able to deliver neutral steric-block ON analogs (PNA, PMO) at micromolar concentrations as attested by redirection of the splicing machinery in both in vitro and in vivo assays. However, mechanistic studies indicate that most of the transfected ONs remain entrapped in endocytic vesicles thus leaving room for much improvement.

Ongoing efforts are geared towards identifying CPPs with improved endosomal escape and at setting up assays to monitor this limiting step in ON-CPP intracellular trafficking. The potential of CPP oligomerization and of CPP derivatization with fatty acids chains will be reported. We also propose liposome leakage to monitor the membrane destabilization potential of CPPs and as a potential convenient assay to model endosome escape.

pHLIP technology for imaging and drug delivery

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We have discovered a peptide, pHLIP (pH Low Insertion Peptide), that targets cells in the acidic tissues that result from a range of pathological states, including tumors, and that can also translocate cell-impermeable cargo molecules across cell membranes in a pH-dependent manner. pHLIP has three major states in equilibrium: soluble in water at normal pH (state I), bound to the surface of a membrane near neutral pH (state II, type I and II), and inserted across the membrane as an α-helix at mildly acidic pH (state III). A drop of pH leads to the protonation of two Asp residues in the transmembrane region, increasing peptide hydrophobicity, which results in the insertion and formation of a transmembrane α-helix (state III), the process which is accompanied by an energy release of about 1.8 kcal/mol. The insertion of pHLIP into a membrane is unidirectional: the C-terminus goes inside a cell or vesicle, and the N-terminus stays outside. The energy released during folding and insertion can be utilized for the translocation of a passenger molecule across a membrane. pHLIP possesses dual delivery capabilities: it can inject and release cargo molecules into the cytoplasm and/or it can tether cargo molecules to the cell surface. In the first scenario, a cargo molecule is attached to the pHLIP C-terminus via a cleavable S–S bond while in the second it is conjugated to the N-terminus via a non-cleavable bond.

pH and thermoresponsive hydrogels of a novel class of *N*-acyl peptides. Characterization, drug encapsulation and release study

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Hydrogels of low-molecular-weight gelators (LMWGs) that can reversibly respond to external stimuli, such as pH, heat, light, and salt have attracted tremendous attention in the recent literature because of their potential applications in pharmaceutical industry as soft materials for drug delivery and tissue engineering. The stimuli-responsive property is also very appealing in the field of biological applications because of the specific pH requirements for many biological systems. For these applications biocompatibility of the gel-forming materials is very important. Various LMWGs capable of forming hydrogels have been reported in the literature. Among these peptide-based gelators have gained importance because of biocompatibility of the constituent materials (amino acids).

In this presentation, gelation behavior of a series of peptide-based amphiphilic gelators, *N*-(4-n-alkyloxybenzoyl)-L-carnosine, in water will be discussed. These hydrogelators have reasonably high gelation numbers at neutral pH. The gelation number decreased when pH was decreased to 2.0. At any given pH, an optimum value of gelation number and gel melting temperature was observed with the C16 amphiphile. These two parameters were found to be sensitive to pH change. Being physical gels all the hydrogels exhibit thermoresponsive behavior; they were found to have good thermal stability and mechanical strength. However, thermal stability decreased upon

addition of salt. The hydrogels were characterized using techniques, such as SEM, XRD, and rheology. The release kinetics of some encapsulated prescription drugs has been investigated. Hydrophilic drugs were observed to be released faster than hydrophobic drugs. Biocompatibility and toxicity of the gelators were also evaluated.

Peptides: chemistry and biology

Peptides from the Sea as source of new drugs

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Peptides natural products are an important source for potential drug candidates. Some of these peptides show a heterodetic structure with disulfide, ester or thioester bonds together with the peptide bond. Depsipeptides, such as Kahalalide F, and Aplidine (both at clinical phase II for several cancers) or Romidepsin and thiodepsipeptides, such as thiocoraline show very often poor pharmokinetic properties. Substitution of the O or S by NH lead very often to an important loss of the activity as it has been shown in Triostin A, another depsipeptide, and very recently in Thiocoraline.

Herein, the NMe amide as the best isostere for the ester and thioester bond will be proposed. Thus, several strategies will discussed for the synthesis of oxa-, aza-, and NMeaza-thiocoraline, which involve the use of a myriad of classical protecting groups (Fmoc, pNZ, Alloc, Boc, Trt) as well as the new one by conformationally restricted mobility. Furthermore, optimization of the coupling steps using phosphonium and aminium HOAt-based, or even DIC with the less reactive HOSu will be described. The main conclusion of the present work is that replacement of a thioester bridge by a *N*Me amide in Thiocoraline results in a compound with nanomolar activity.

Furthermore, a new topological concept will be introduced: *Siamese peptides*, which are cyclic peptides that share a bond. This concept will be illustrated taking as model the depsipeptide Sansalvamide.

Finally, the use of nanotechnologies for drug delivery to overcome the problems associated with the poor pharmacokinetic properties of natural product based drugs will be discussed.

Diversity-oriented solid-phase synthesis of cyclodecapeptides on the scaffold of natural laterocidin for antibiotic screening

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Cyclopeptides represent a large and interesting class of bioactive molecules displaying a broad spectrum of significant biological activities. Synthesis of head-to-tail cyclopeptides has attracted a considerable interest since the antibiotic gramicidin S was found to be a cyclodecapeptide. Many antibiotics and toxins are also known to be cyclopeptides. Due to the growing interest that cyclic peptides have generated, intense efforts have been invested in developing efficient methods for peptide cyclization.

Laterocidin is a novel cyclodecapeptide antibiotic isolated from laboratory cultures of a bacterial strain (*Brevibacillus laterosporus* VKPM B-8287). It exhibits distinctive potent antibiotic activities among the family of cyclopeptide antibiotics that include gramicidin S, tyrocidines, streptocindins and loloatins. To facilitate the structure-activity relationships study, novel and facile synthetic methods are highly desirable although these natural products have been synthesized by traditional methods. Based on structure characteristic of natural laterocidin, here we report a novel method for total solid-phase synthesis of laterocidin and its analogues by using Dmab group as a temporary α-COOH protecting group during solid-phase synthesis with Fmoc chemistry. This synthetic method for the natural products will not only expedite the elucidation of the structure-activity relationships, but also significantly facilitate the optimization of their therapeutic index for containment of microbial resistance.

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Antifreeze glycopeptide analogs: synthesis, structural, and functional analysis

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Biological antifreezes enable life in subpolar seas at water temperatures as low as −1.9°C. There are four classes of structural diverse antifreeze proteins (AFPI-IV) and a single class of antifreeze glycoproteins (AFGP), the latter being further divided into eight subtypes according to the covered mass fraction. In case of AFGP, little is known about the molecular mechanism of antifreeze activity due to the tedious purification from natural sources and challenging synthesis of heavily glycosylated peptides.

All AFGP consist of a varying number of —(Ala-Ala-Thr)$_n$— (n = 4–50) with every threonine side chain being glycosylated with β-D-galactosyl-(1-3)-α-*N*-acetyl-D-galactosamine. This pattern is highly conserved among different species. Minor sequence variations where alanine is substituted by proline or the glycosylated threonine by arginine are biologically active. The proteins and peptides initiate thermal hysteresis, change the crystal habit and suppress recrystallization and heterogeneous ice nucleation. Although essential moieties of the peptides are known, the protein/peptide-ice interface is not yet understood. Especially, the account of sequence variations has barely been elucidated. Solid phase peptide synthesis enables the preparation of peptides containing proline and other structure inducing amino acids. The synthesis has been accomplished successfully using HATU activation and microwave-assistance during the cycles. The peptides have been structurally analyzed by NMR and CD. Furthermore, their activity has been tested by examining the recrystallization inhibition.

A protein chimera with a beta/gamma-motif mimicking alpha-helical turns

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The complex behaviour of proteins has inspired widespread interest in unnatural biopolymers that mimic certain folding motifs and have protein-like functions. The main advantage of such artificial peptides would be their stability towards proteolytic degradation which is a key feature in the development of peptide-based drugs. One way to derive such compounds is the homologation of the α-amino acid backbone to β- and γ-amino acids. Investigations on such oligomers have shown their enormous potential for secondary structure design. Our main goal is to generate synthetic foldamers based on β/γ-hybrid peptides that fold into stable helices.

According to ab initio MO studies hybrid peptides composed of alternating β- and γ-amino acids are very well suited to adopting α-helical coiled coil-like conformations. We selected a α-helical coiled coil forming sequence as the basis peptide. This peptide carries exclusively Lysine residues in e and g positions of the *heptad* repeat. A subset of α- amino acids within the middle part of B was replaced with an alternating sequence of β- and γ-amino acids to generate α/β/γ-hybrid peptides B(3β2γ) and B(5β4γ). A second coiled coil-based peptide was designed as interaction partner for B, which exclusively presents Glutamic acid residues in positions e and g of the *heptad* repeat.

The interaction potential of the hybrid peptide B with the natural sequence A as well as the stability of hetero-oligomer formation was investigated by CD spectroscopy. In addition, FRET experiments provide evidence of heterogeneous helix formation. The oligomerization state was studied by analytical ultracentrifugation.

Nucleopeptides with predictable 3D-architectures

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Polyamide backbones with nucleo-bases as pendants have gained much attention in view of their potential applications in antisense therapy and immunotherapy. Indeed, such hybrid molecules are insensitive to phosphatase hydrolytic actions, while retaining (or even improving) polynucleotide complexation.

By taking advantage of our long-standing experience in the synthesis and 3D-characterization of conformationally constrained alpha-amino acids and peptides, particularly those based on C-alpha-tetra-substituted α-amino acids, we designed and synthesized short, beta-turn- or helix-forming alpha-nucleo-peptides. Nucleo-base-containing residues (beta-substituted Ala) were inserted into host, homo-peptide stretches of Aib (alpha-aminoisobutyric acid). The primary structure of the longest peptides were designed in order to allow all nucleo-bases to be aligned on the same face of the helical molecules. These new nucleopeptides are characterized by (1) markedly rigid backbones, (2) helical conformations (usually of the 3–10 type) and (3) high predictability of their 3D-structures. Thus, they might be able to force a complementary polynucleotide chain to adopt unusual conformations

Nucleopeptides with a lower percentage of Aib residues were also synthesized. Interestingly, they are able to cross the biological membranes without displaying any significant cytotoxicity. In addition, they exhibit a remarkable resistance to the enzymatic degradation. Therefore, we believe these molecules are promising candidates for the development of new drugs.

Synthetic mimicry of the CD4-binding site of HIV-1 gp120 for the design of immunogens

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Entry of the virus HIV-1 into T-lymphocytes is initiated by interaction of the HIV-1 envelope glycoprotein gp120 with the cellular receptor CD4. The binding site of gp120 for CD4 (CD4bs) represents a conserved region in this otherwise highly variable protein. Furthermore, the epitope of the broadly neutralizing anti-HIV-1 antibody mAb b12 has been found to overlap the CD4bs. Synthetic mimetics of this binding site are therefore promising immunogen candidates for the elicitation of virus-neutralizing antibodies.

Based on the X-ray crystal structure of core gp120 in complex with two extracellular CD4 domains, we have designed and generated a range of scaffolded peptides, which present three gp120 fragments that make up its discontinuous CD4bs. Some of these peptides were found to compete with gp120 for binding to CD4 and mAb b12, respectively.

Polyclonal antisera raised against a CD4bs mimetic peptide were shown to recognize, with a specificity related to that of mAb b12, gp120 in its native trimeric conformation, as well as complete, membrane-bound trimeric HIV envelope protein. Ongoing studies addressing the issue of structural mimicry of the CD4bs of gp120 by synthetic peptides, will guide the design of improved synthetic mimetics of the CD4 binding site of gp120 as immunogen candidates for the elicitation of broadly neutralizing anti-HIV-1 antibodies.

Modified cryptophycins

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Cryptophycins are a class of 16-membered macrocyclic depsipeptides. Cryptophycin-1 was isolated in 1990 from cyanobacteria. Several natural and artificial compounds of this class proved to be extremely cytotoxic. The bioactivity is based on their ability to interact with tubulin. They display striking antiproliferative activities both in vitro and in vivo with 100- to 1,000-fold increased potency compared to paclitaxel and vinblastine. Cryptophycins are also remarkably cytotoxic against several multidrug-resistant tumor cell lines and solid tumors. Cryptophycin-52 entered phase II clinical trials but failed due to its high neurotoxicity.

As a consequence, our research group focuses on the synthesis of structurally modified cryptophycins. Recently, we also published a short and efficient synthesis of a cryptophycin unit A building block and are now able to present an even shorter synthesis of a new unit A building block via a modified Shi-epoxidation giving rise to a *cis*-epoxide in cryptophycin. So far, such a stereochemistry within the unit A fragment had only been available by altering the stereochemistry of cryptophycin-1 on small scale.

New orthogonally photocleavable thiol protecting groups in peptide chemistry

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Photolabile protecting groups have numerous applications in chemistry and in biology as well. Their removal only requires light and allows a very mild deprotection of sensitive molecules. Therefore, the protection of biological signalling molecules by conversion into inactive light-sensitive derivatives is very attractive, because they permit the controlled delivery of bioactive molecules without physically disturbing within organised biological systems with high temporal and spatial precision. Furthermore, photolabile groups are valuable tools to expand synthetic strategies in organic chemistry allowing for protecting COOH, OH, NH_2, and SH functions. Being stable towards acidic and basic conditions, the well-known 2-nitrobenzyl (NB) and 4.5-dimethoxy-2-nitrobenzyl (DMNB) protecting groups have been employed in organic synthesis. Due to improved water solubility the α-carboxy-2-nitrobenzyl (CNB) group and its α-carboxy-4.5-dimethoxy-2-nitrobenzyl (CDMNB) analogue extend the range of applications. The photoefficiency is clearly higher for the *S*-α-carboxy-nitrobenzyl protected derivatives than for the corresponding groups without α-carboxylate substituent. Comparing CNB and CDMNB as protecting groups for cysteine residues we found a clearly better photoefficiency for CDMNB and products of higher purity. With respect to the photodecarboxylation as potential side-reaction during photocleavage of CNB and CDMNB groups we could confirm an accelerating effect of amine but we found this being not the only cause of photodecarboxylation.

The development of the {7-[bis(carboxymethyl)amino]coumarin-4-yl}methyl (BCMACM) –type protecting group and its application in conjunction with CNB-type groups facilitates the selective removal of the two groups from different cysteine residues in a molecule which will be demonstrated for resect, a peptide involved in chemotaxis of *A. punctulata* sperm.

Synthesis and inhibitory potency of Orn and DAP analogs towards bacterial enzymes

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A series of N^{α}-acyl (alkyl)- and N^{α}-alkoxycarbonyl-derivatives of L- and D-ornithine were prepared, characterized and analyzed for their inhibitory potency towards the bacterial enzyme N^{α}-acetyl-L-ornithine deacetylase (*ArgE*). Most of the compounds tested provided IC_{50} values in the μM range, indicating that they are moderately strong inhibitors. N^{α}-chloroacetyl-L-ornithine was the best inhibitor tested towards *ArgE* providing an IC_{50} value of 85 μM while N^{α}-trifluoroacetyl-L-ornithine, N^{α}-ethoxycarbonyl-L-ornithine and N^{α}-acetyl-D-ornithine weakly inhibited *ArgE* activity providing IC_{50} values between 200 and 410 μM. Weak inhibitory potency towards *Bacillus subtilis* for N^{α}-acetyl-D-ornithine and N^{α}-chloro-, N^{α}-dichloro- and N^{α}-trichloroacetyl-ornithine was also observed. These data suggest that the N^{α}-substituted ornithine derivatives are capable of getting across the cell membrane and that *ArgE* is the bacterial enzymatic target. In this study, also a series of N^{α}-acyl-derivatives of racemic diaminopimelic acid (DAP) was prepared using a "double" orthogonal protection to obtain functional selectivity for mono-*N*-acylation, only. Surprisingly, they showed no potency towards the bacterial enzyme N^{α}-succinyl-L,L-diaminopimelic acid desuccinylase (*DapE*) and only weak potency towards *ArgE*.

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Discovery of neurotrophin mimetics: synthesis and biological activity of NGF agonists

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The neurotrophins (NGF, BDNF, NT3/4) and their receptors have been proposed as therapeutic tools in a wide range of pathologies ranging from neurodegenerative disorders such as Alzheimers Disease, Parkinsons Disease to cancer. Nevertheless, neurotrophins themselves share poor pharmacokinetic profiles because of their sensitivity to proteolysis, restricted penetration of the blood–brain barrier and expensive production, limiting their use as drugs. Therefore small molecules able to interact with the neurotrophin receptors, either as agonists or antagonists, are of great interest for many therapeutic applications and could represent the suitable solution for stability to proteolysis and specificity in receptor binding, the two main concerns in drug design.

The potential applications of such small molecules could be the treatment of acute or chronic neurological disorders (such as stroke and Alzheimer disease), cutaneous pressure ulcers, and corneal neurotrophic ulcers. We have recently reported on a new classes of conformationally restricted peptidomimetics. These compounds were modified to mimic the loop 4 residues of NGF, thus having the possibility to interact with the NGF-receptor TrkA. The use of biosensors and in vitro and in vivo tests demonstrated that these new peptidomimetics act as pure agonists of NGF at nanomolar concentration level.

Modification of small peptides: one-pot conversion of alpha-amino acids into amino acid analogues

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An efficient methodology is reported for the direct transformation of alpha-amino acids in peptides into amino acid analogues, such as beta-amino aldehydes, beta-amino esters or alpha-amino phosphonates. The procedure couples a decarboxylation reaction and the introduction of other functional groups. The process is operationally simple, and saves materials and time, since no purification of the intermediates is needed. The mild reaction conditions are compatible with most functional groups. Finally, this versatile methodology allows the preparation of a variety of hybrid peptides.

Studies of an active peptide isolated from *Hyla biobeba* and its synthetic analogues

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The development and the spread of bacterial resistance has become in the major public health problem. As result, the research and studies of novel bioactive molecules or modification of the existent ones has been crucial for future drug development. In this way, isolated toxins from frog skin secretion can be considered an important source for study of new biologically active compounds. Castro and co-workers extracted the peptide Hy-b3 (GLLSTVGGLVGGLLNNLGL) from the frog *Hyla biobeba*, which showed considerable antimicrobial activity. The aim of this work was to supply information regarding the structure/function of this peptide by conformational and biological assays. In this context, the wild type peptide (WT) and three analogues with Leucine residues substituted by Tryptophan in the 3, 9 and 17 positions (W3, W9 and W17 respectively) were synthesized by SPPS methodology using Fmoc chemical approach. The peptides were characterized by HPLC, amino acid analysis and mass spectroscopy. Conformational properties were investigated by CD techniques in water, TFE and in zwitterionic micelles (LPC). The CD experiments demonstrated that in water, the peptides have a random structure, although in TFE and LPC solutions they acquired an ordered structure, composed mainly by α-helix. WT and W17 peptides showed antifungal activity (MFC = 125 μg/mL), however when Leucine was substituted in the positions 3 and 9 (W3 and W9), the activity increased considerably. On the other hand, the hemolytic activity for all peptides was insignificant in concentrations lower than 256 μM.

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Synthetic study on prion protein fragments using a SPPS and native chemical ligation

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Prion proteins are suspected as causative agents of several neurodegenerative diseases such as CJD and BSE. However, their function and decision between viral and protein-only hypothesis have not been fully solved, yet. Although recombinant prion protein is available, it is not easy if possible to introduce some specific modifications at site of interests. Developing a route to fully synthetic prion protein is therefore highly desirable. Its molecule of more than 200 amino acid residues requires syntheses of shorter segments, which are finally linked together. The suitable segments prepared by SPPS can be also applicable as a tool for physico-chemical studies of the prion space structures and folding.

In the mouse prion (MoPrP) synthesis, we started with MoPrP(203-231) to study a native chemical ligation between Cys and Met in the positions 212 and 213 of corresponding peptides, with respect to effects of electron withdrawing and electron donating groups. An alternative ligation sites at Ala and Val residues were also tested using selective desulfurization of before introduced Pen and Cys residues by metal-based (Raney nickel) or metal-free (VA-044) procedures. The solution conformation of several overlapping segments of PrP sequence was estimated on utilization of chiroptical procedures.

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Design and synthesis of *N*-acylpeptides for screening antibiotics against clinical drug-resistant microbes

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The emergence in all regions of the world of strains of pathogenic bacteria and fungi with resistance to commonly used antibiotics constitutes a potentially serious threat to public health and has necessitated a search for novel types of antimicrobial agent to which the microorganisms have not been exposed. Acylpeptides are unique among the antimicrobial peptides in that they are relatively smaller in size as compared to other cationic antimicrobial peptides, and yet, exhibit excellent antimicrobial activity. Fatty acid acylation of antimicrobial peptides of bacterial and fungal origin have mostly been limited to non-gene-encoded peptides from Pseudomonas syringae. Short acylpeptides are monomeric insolution while longer ones form oligomers and this feature can potentate the killing of microbes. Their mechanism of action is mediated by direct disruption of membrane electric potentials with less likelihood for development of cross-resistance. However, it is not clear if acylation influences the association of hydrophilic portions of peptides with membranes or modulates the orientation of peptides in membranes. Therefore, it would be relevant to study the interactions of antimicrobial acylpeptides and anionic model membranes as it would help in understanding the biophysical properties governing the association of fatty acylated peptides with biological membranes. Here, we designed and synthesized 24 fatty *N*-acylated peptides for screening antibiotics against clinical drug-resistant microbes. The results indicated that RCO-K-K*-K-K*-K-K*-K ($R = C_{11}H_{23}$, $C_{13}H_{27}$) and RCO-K-K-K-K($R = C_{13}H_{27}$, $C_{15}H_{31}$) among the 24 synthetic acylpeptides, possesses the most excellent antimicrobial activities against clinical multidrug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (Gram-positive) and quinolone-resistant *Escherichia coli* (Gram-negative).

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Analysis of retinas of cystathionine β-synthase (*cbs*) deficient mice: a model of hyperhomocysteine-induced retinopathy

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Hyperhomocysteinemia is implicated in glaucoma, a blinding disease in which retinal ganglion cells (RGCs) die. Earlier, we reported that cultured RGCs die when exposed to excess homocysteine. To determine consequences of sustained retinal hyperhomocysteinemia in vivo, morphological, biochemical and molecular analyses were performed in retinas of *cbs* mice. Retinal levels of homocysteine were analyzed by HPLC and revealed ~twofold elevation in retinal

homocysteine in $cbs^{+/-}$ mice and ~sevenfold elevation in $cbs^{-/-}$ mice. Systematic morphometric analysis was performed on mouse retinal cryosections at 5, 15, and 30 weeks and revealed ~20% loss of RGCs in $cbs^{+/-}$ mice by 5 weeks and decreased thickness of inner plexiform and nuclear layers by 15 and 30 weeks, respectively. Microarray analysis was performed using RNA isolated from neural retinas of $cbs^{+/+}$ and $cbs^{+/-}$ mice. Of 36,212 genes screened, there was a ≥twofold change in expression of 1,216 categorized into six functional groups. Of particular interest were changes in: (1) pro-apoptotic: *Bat3* (↑2.3-fold), (2) anti-apoptotic: *Survivin* (↓2.8-fold), (3) cell cycle: *Egr1* (↑ 4.7-fold), (4) antioxidant: subunits of *GST* (↓2.0- to-5.2-fold) and *SOD3* (↓2.6-fold), (5) calcium signaling: *Slc24a4* (↑3.4-fold), (6) axon growth/guidance: *Efnb3* (↑2.6-fold), *Ablim2* (↑2.66-fold), *Tsc2* (↑2.5-fold). In conclusion, retinal homocysteine is elevated in $Cbs^{+/-}$ mice, which is associated with significant loss of RGCs and decreased thickness of inner retinal layers. Altered expression of genes related to apoptosis, axon guidance, cell cycle, and antioxidant levels lay the foundation for future studies to determine the cause of the retinal phenotype in this model of retinal hyperhomocysteinemia.

Synthesis of Nα-hydrazino- and Aza-peptoids based on substance P: C-terminal fragments and their trypsin inhibitory effect

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Serine protease inhibitors (Serpins) is a group of proteins with similar structures, which were first identified as a set of proteins able to inhibit proteases function. The human plasma proteins antithrombin and antitrypsin, which play key roles in controlling blood coagulation and inflammation, respectively, were the first members of the serpins superfamily to be extensively studied. Trypsin-like serine proteases are essential for many biological processes. Because of this a large number of synthetic peptides have been designed and synthesized, based on the structure of inhibitors, active against trypsin or chymotrypsin.

In the present work we have synthesized a series of N^{α}-hydrazino-peptoids and aza-peptoids and studied their trypsin inhibitory effect. These peptidomimetics are expected to show enhanced metabolitic stability and bioavailability in comparison with natural parent peptidic analogs. Their structures are the following:

N^{α}-Hydrazino-peptoids

1. H_2N-N(Bzl)-CH_2-CO]^1-D-Trp^2-Leu^3-OH
2. Glp^1-NPhe^2-Gly^3-[NH-N(Bzl)-CH_2-CO]^4-D-Trp^5-Leu^6-OH
3. Glp^1-NPhe^2-Gly^3-[NH-N(Bzl)-CH_2-CO]^4-D-Trp^5-Leu^6-OH
4. Glp^1-Glu(Bzl)^2-NPhe^3-Gly^3-[NH-N(Bzl)-CH_2-CO]^5-D-Trp^6-Leu^7-Glu(Bzl)^8-NH_2

Aza-peptoids

5. H-D-Trp^1-[NH-N(Bzl)-CO]^2-D-Trp^3-OH
6. H-D-Trp^1-[NH-N(Bzl)-CO]^2-D-Trp^3-Leu^4-OH
7. Glp^1-NPhe^2-Gly^3-D-Trp^4-[NH-N(Bzl)-CO]^5-D-Trp^6-OH

All the syntheses were carried out stepwise by SPPS, using the Fmoc/Bu^t methodology on the solid support 2-chlorotrityl chloride resin and DIC/HOBt as coupling reagent. The products were purified (HPLC) and identified (ESI-MS). Their inhibitory effect against trypsin activity has partly measured, while other compounds are under investigation.

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Plant amino acids

High-lysine maize lines expressing a new protein in the endosperm

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Cereal crops represent approximately 50% of the plant protein consumed worldwide, however, cereal seeds are nutritionally deficient in important amino acids such as lysine and tryptophan, a situation that has presented researchers with the major task of improving the nutritive value of cereal seeds. Aspartate is a precursor for two main pathways, the first one leading to the synthesis of asparagine. Aspartate is also the common precursor of the essential amino acids, lysine, threonine, methionine and isoleucine. Since the late 1970s research groups have tried to produce cereal crops containing high lysine concentrations in their seeds. Several strategies have been attempted: (1) plant breeding programs, which requires a long time to achieve some progress; (2) natural occurring mutants, such as the opaque-2 maize and the hiproly barley mutants; (3) biochemical mutants exhibiting altered regulation of key the enzymes controlling lysine metabolism; (4) plant transformation: transgenic plants expressing altered enzymes or bacterial enzymes; and (5) plant transformation: transgenic plants with altered storage protein distribution. We started a new project in which we have produced maize transgenic lines overexpressing zeolin (phaseolin [bean] + γ-zein [maize]) protein using the endosperm specific γ-kafirin (sorghum) promoter. Such a protein contains several lysine residues. Almost 100 positive plants exhibited between 70 and 160% increase in the prolamin fraction with up to 2.6-fold increase in the zein 1 storage protein fraction, in which the zeolin protein is included. We are currently performing agronomic tests with the most promising lines.

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Functional genomics approaches to study ammonium nutrition and amino acid biosynthesis in conifers

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Conifers are the most important group of gymnosperms, which include tree species of great ecological and economic importance that dominate large ecosystems and play an essential role in global carbon fixation. Functional genomics studies are needed to understanding fundamental conifer biology and adaptation of conifer trees to environmental

conditions. Our laboratory has been involved for more than 15 years in the study of conifer metabolism by using a variety of experimental approaches and including biochemical, immunological, recombinant-DNA techniques, and more recently transcriptomic and proteomic approaches. Expression analysis as well as cellular and subcellular localization have been used to assess the role of genes/enzymes involved in amino acid biosynthesis and the reallocation of nitrogen from the seed towards photosynthetic and lignified tissues of young trees. New adapted methods for the precise localisation of mRNAs and proteins in conifers have been applied and this experimental approach has provided new insights on how metabolic pathways are organized in different cell types. Transcriptomic and other functional analysis have been used to examine the response to nitrogen nutrition and how changes in primary metabolism can influence tree growth and developmental programmes. We have isolated the promoters of several pine genes and identified regulatory sequences able to interact with specific factors regulating transcriptional activity. An overview of this research programme will be presented and discussed.

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Bioinformatic dissection of gene modules controlling the operation of plant amino acid metabolic networks

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We have recently developed a new bioinformatics tool adapted for analyzing the response of *Arabidopsis thaliana* genes controlling plant metabolism to abiotic and biotic stresses. Using this new approach to analyze publicly available microarray datasets, we have identified novel expression coordination patterns between gene modules controlling the operation of central amino acid metabolic networks in response to the various abiotic and biotic stresses. We have also further developed this bioinformatics tool to elucidate the transcriptional response of genes encoding the entire set of Arabidopsis metabolic enzymes to the various stress conditions. We will present the results of this study, which: (1) elucidate several novel regulatory principals of plant metabolism; and (2) can be used to elucidate regulatory genes that control the operation of plant metabolism.

Sulfur amino acids and redox homeostasis in Arabidopsis

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Cysteine and glutathione are present in many compartments of plant cells, including the organelles, cytosol, ER and possibly cell wall and vacuole. Synthesis of cysteine and glutathione in Arabidopsis is distributed between the cytosol and the organelles and implicates a number of membrane transport processes. Exchange of glutathione between the compartments is implicated by the sole presence of glutathione reductase (GR) in plastids, mitochondria and cytosol, unless other proteins would mediate electron transfer to oxidized glutathione. The membrane transport mechanisms may occur as reduced, oxidized, conjugated glutathione or similar to the glutathione cycle found in animals. To investigate the role of cysteine and glutathione synthesis as well as glutathione reduction by GRs in the three compartments, T-DNA insertion lines of the corresponding genes of Arabidopsis were characterized. With respect to glutathione reduction GR2 encodes plastid and mitochondrial GR by way of a dual target sequence and a null allel of *gr2* was lethal. Inactivation of *gr1* encoding cytosolic GR1 caused no visible phenotype, but resulted in 60% reduction of overall GR activity and significantly increased contents of oxidized glutathione but also total glutathione, indicating enhanced flux through cysteine. To dissect the roles of both organelles in redox homeostasis the *gr2* mutant was complemented by plastid or mitochondria-specific constructs. Expression of GR1 only in plastids was sufficient for survival while targeting only to mitochondria was not. Thus, exchange of reduced or oxidized glutathione or of precursors across the plastid membrane was insufficient to meet the demands inside the organelle. This demand for plastidic glutathione reduction capacity was already essential during early embryo development.

'Systems' biology of inter- and intracellular metabolite transport in plants

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Metabolic processes are regulated in a co-ordinate manner to balance macro- and micronutrients in possible synergistic or antagonistic effects. The high degree of compartmentation on plant and cellular level enhance the need for a good coordination of metabolic precursors, intermediates, and end products between plant organs and cellular compartments. As plants are limited in there mobility, they developed mechanisms to overcome local limitations of nutrients in their direct environment. Plants respond, e.g. by activation of lateral root formation into nutrient enriched patches to overcome nutrient limitations. On the other side metabolite synthesis is in several cases not restricted to one compartment indicating cooperation between the participating compartments. Factors regulating these processes are so far not well described being involved in the regulation of uptake, transport and compartmentation of metabolites to keep the metabolite homeostasis of, e.g. amino acids.

To this end, we make use of biochemical, genetic, physiological, and statistical techniques to investigate transport and compartmentation processes in plants. Data will be presented giving new insights into the regulation of cysteine biosynthesis on plant and cellular level.

Amino acid metabolism as an entry point to secondary metabolism

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Plants produce chemically-diverse secondary metabolites derived from amino acids. In general, primary metabolites including amino acids have fundamental functions in every aspects of life, and hence, primary metabolism is regulated so as to be robust against environmental perturbation. On the other hand, a major role of secondary metabolism is to supply the dead-end products which have specific

biological functions (for example, insect repellents, UV protectants, and cryoprotectants) in response to environmental stimuli. Hence, secondary metabolism is presumably regulated so as to respond dynamically to environmental changes. Our omics-based study using Arabidopsis revealed the genes involved in methionine-derived glucosinolate biosynthesis. Modulation of the expression of these genes affected the amino acid contents, suggesting complicated relationship between amino acid metabolism and secondary metabolism. In this presentation, our recent results on amino acid metabolism will be introduced.

Glutamate: glyoxylate aminotransferase (GGAT) functions as a regulator of amino acid content in *Arabidopsis*

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In photorespiration, peroxisomal glutamate:glyoxylate aminotransferase (GGAT) catalyzes the reaction of glutamate and glyoxylate to produce 2-oxoglutareate and glycine. In other words, the GGAT reaction links the photorespiratory carbon and nitrogen cycles and may function as a plant-specific regulator of amino acid content. However, the gene encoding GGAT has not been identified. Here, I intended to identify the GGAT gene and to examine the importance of the GGAT gene product. Based on the analysis of knockout line of alanine:2-oxoglutarate aminotransferase like gene, we succeeded to identify the *GGAT* gene. A phenotypic analysis indicated that the knockout line (*ggat1-1*) exhibited a well-known photorespiratory-deficient mutant phenotype; growth repression was observed and plants recovered under high CO_2 or low light conditions. To understand the role of GGAT in the regulation of amino acid levels, we generated and characterized plants overexpressing the *GGAT1* gene. The pool size of serine and glycine—products of the GGAT reaction—increased markedly in all *GGAT1* overexpression lines. Further, the levels of these amino acids were strongly correlated with the levels of *GGAT1* mRNA and GGAT activity. Air (CO_2 concentration), light, and nutrient conditions affected the amino acid profiles in the *GGAT1* overexpression lines. These results suggested that the photorespiratory aminotransferase reaction catalyzed by GGAT is an important regulator of amino acid content.

Transporters for amino acids and peptides in plants

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Nitrogen is an essential nutrient for plant growth and productivity. Following uptake from the soil or assimilation within the plant, organic nitrogen compounds are transported within and between cells as well as over long distances in support of plant metabolism and development. Research mainly focused on amino acids that represent in most plants the principal transport form for organic nitrogen, but transport of small peptides and other organic nitrogen forms has also been investigated.

Transporters for amino acids and peptides have been identified and characterized using heterologous expression systems, i.e. *Saccharomyces cerevisiae* mutants and *Xenopus laevis* oocytes. These studies identified transporters belonging to different gene families that mediate low and high affinity transport of amino acids or peptides. Results from different labs revealed differential expression of the respective genes and elucidated the importance of individual transporters for uptake of amino acids or peptides from the soil, for long distance transport and translocation to seeds, respectively.

An overview of current knowledge and recent findings will be presented.

Metabolomics-based functional genomics and network analysis for plant amino acid metabolism

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Metabolomics is a powerful approach to decipher genes' function and elucidate metabolic networks in plants. We have investigated the metabolic profiling of the mutants of serine acetyltransferase genes and β-substituted alanine synthase (BSAS) genes, which encode cysteine synthase (CSase) [O-acetylserine (thiol) lyase] and β-cyanoalanine synthase (CASase) of Arabidopsis thaliana. This analysis concluded mitochondrial BSAS3;1 is a genuine CASase, and β-cyanoalanine is present as a conjugated form as γ-glutamyl-β-cyanoalanine. We also investigated the metabolic network of two mutants (methionine-over accumulation 1 [mto1] and transparent testa4 [tt4]). Although the mutants showed no apparent morphological abnormalities, the overall metabolite correlations in mto1 were much lower than those of the wild-type and tt4 plants, indicating the loss of overall network stability due to the uncontrolled accumulation of methionine. In the tt4 mutant, several new correlations were observed, suggesting an adaptive reconfiguration of the network module. Gene-expression correlations presumably responsible for these metabolic networks were determined using the metabolite correlations as clues.

Function of glutamine synthetase and glutamate synthase in rice plants

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A major source of inorganic nitrogen for rice plants grown in paddy soil is ammonium ions. The ammonium-ions, actively taken up by the roots, are assimilated into the amide-residue of glutamine (Gln) by the reaction of glutamine synthetase (GS) in the roots. The Gln is converted into glutamate (Glu), which is a central amino acid for synthesis of a number of amino acids, by the reaction of glutamate synthase (GOGAT). Although a small gene family for both GS and GOGAT is present in rice, ammonium-dependent and cell-type specific expression suggest that cytosolic GS1;2 and plastidic NADH-GOGAT1 are responsible for the primary assimilation of ammonium ions in the roots. In the plant top, approximately 80% of the total nitrogen in the panicle is remobilized through the phloem from senescing organs. Thus, nitrogen remobilization determines productivity of rice. Since the major form of nitrogen in the phloem sap is Gln, GS in the senescing organs and GOGAT in developing organs are important for nitrogen remobilization and re-utilization, respectively. Our recent work with a knock-out mutant of rice clearly showed that GS1;1 and NADH-GOGAT1 are both responsible for the remobilization processes. Since the integration of the functions of many genes is required for the overall process of nitrogen utilization in plants, it is not easy to draw the whole picture from studies on an individual gene. Recent studies obtained from the profiling of metabolites in the *OsGS1;1* mutants, together with transcriptome analysis, will be discussed in this symposium.

Renewable clean energy resources in Egypt

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World now is in an increasing demand for new energy resources. We hope that these will be clean, renewable, and cheap resources. In the past years, there were great efforts to use natural substances that contain a high starch and sugar content for the production of ethanol as a pure and clean energy resources which was named the biofuel or biodiesel. The irony is that industrial countries, in order to overcome the storage in energy resources, have devoted efforts to utilize a number of foodstuffs, the edible crops for human, namely: wheat, corn, maize and rice as raw materials for producing fuel. In our research proposal, we introduce a group of natural resources, non edible, to produce the biofuel namely, Jatropha, Seaweeds and Balanites. Besides we propose other uses for these sources. A wide area of land will be cultivated with Jatropha, then it will be processed for the production of Biofuel. Seaweeds will be harvested from the Egyptian seashores and Balanites will be collected from the Egyptian markets for the production of biofuel and the other uses.

Characterization of aminoaldehyde dehydrogenases from pea and tomato

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Aminoaldehyde dehydrogenases (AMADH, EC 1.2.1.19) oxidize omega-aminoaldehydes arising from polyamine degradation. Based on their amino acid sequences, the enzymes belong to the same group as betaine aldehyde dehydrogenases (BADHs, EC 1.2.1.8), which participate in plant response to osmotic stress. Two isoenzymes from *Pisum sativum* (PsAMADH1 and PsAMADH2) and two isoenzymes from *Lycopersicon esculentum* (LeAMADH1 and LeAMADH2) were expressed in *E. coli* and subsequently purified to homogeneity by immobilized metal ion affinity chromatography and ion-exchange chromatography. Enzyme kinetics was performed with all recombinant enzymes. The obtained results show that PsAMADH2 has much better affinity to the best substrate 3-aminopropanal compared with PsAMADH1 and that the compound is also oxidized more efficiently. Interestingly, all enzymes, but especially LeAMADH1, are able to oxidize pyridine carboxaldehydes and some other heterocyclic aldehyde compounds in addition to omega-aminoaldehydes and aliphatic C3 to C7 aldehydes. Also various NAD^+ analogs functioned as effective coenzymes. To get insight into AMADH catalysis and specificity, site-directed mutagenesis of PsAMADH2 has been performed together with kinetic characterization of the mutants. Both pea AMADHs were successfully crystallized with and without NAD^+. X-ray data were collected up to 2.4 Å resolution for PsAMADH1. Molecular replacement using human aldehyde dehydrogenase as a template confirmed that recombinant PsAMADH1 is a dimer. The crystal structure is currently under refinement.

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Amino acids metabolism and storage proteins of maize endosperm mutants

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Little information is available about maize mutants that have been classified as high-lysine and exhibiting the opaque phenotype, such as opaque (o) mutants 1, 5, 6, 7, 9, 10, 11, 13 and 14 among others, and the floury (fl) mutants 1, 2 and 3. We have carried out a detailed biochemical characterization of these mutants. The information obtained has included total protein, nonprotein N, soluble amino acids, albumins/globulins, zeins and glutelins together with the enzymes involved in lysine biosynthesis and degradation during endosperm development. The o2 mutant exhibited the highest relative concentration of free lysine followed by the fl2 mutant, whereas the o11, o5 and fl3 exhibited lower relative concentrations of free lysine when compared to their wild-type counterparts. Based on 2D-PAGE analysis of zeins, 52 zein polypeptides were detected among the 14 genotypes tested. In general, the mutations decreased the number of zein isoforms detected on the 2D gels, indicating a decreased zein amount and diversity. The activity of AK varied considerably among the genotypes, with fl3 being the lowest and the wild-type B37+, the highest. The inhibition by lysine and threonine was also variable. The activity of HSDH was lowest in o13 and highest in the o2 mutant. The enzymes LOR and SDH, both involved in lysine degradation exhibited large variations, particularly for LOR activity. The o2 and fl2 mutants exhibited 6- and 7-fold reductions in LOR activity, when compared to the wild-type maize lines.

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Quality protein maize zein storage protein characterization by 2DE-PAGE

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Cereals typically provide ~50% of the dietary protein for humans and can comprise up to 70% of the protein intake. Maize seeds are characterized by the low contents of lysine, particularly in the most abundant seed storage protein, the zeins (prolamin type). The objective of this study was to compare storage proteins maize seeds, through bidimensional polyacrylamide gel electrophoresis (2DE-PAGE), of a wild type maize line (L161n) and two Quality Protein Maize (QPM) lines (L161o and L161q). These QPM lines are the sixth generation of modified backcross exhibiting different grain vitreous aspects; L161o grains are whole opaque while L161q grains are vitreous-top and opaque-bottom. The storage proteins were extracted and the zein fraction subjected to 2DE-PAGE. Nonlinear pH 3–10 18 cm strips and 12.5% SDS-PAGE was used. The results revealed higher zein concentration in the L161n genotype (1.7- and 1.5-fold) when compared to the L161o and L161q lines, respectively. 2DE-PAGE revealed a smaller number of protein spots in the L161q genotype when compared to the other lines. The L161q genotype also accumulated 1.4-fold higher lysine content than the wild-type L161n. Such results indicated that the QPM lines used have different protein storage pattern, specially with a reduced zein fraction. Such an altered distribution of the

storage proteins resulted in higher lysine content in QPM lines due to the alteration of zein with concomitant increase in the other storage protein fraction which naturally exhibited higher contents of lysine in their structure.
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Cell-penetrating peptides: Trojan horse for macromolecule delivery in plants too?

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Cell-penetrating peptides (CPPs) are a class of short peptides with a property to translocate across mammalian cell membranes and recently reported in plant by three independent groups. Despite fundamental differences between animal cell and plant cell composition, the CPP uptake pattern between the mammalian system and the plant system is very similar. Tat $_{49–57}$ RKKRRQRRR basic domain, one of the shortest known cell penetrating peptide, Tat-2 *p*VEC and transportan internalisation in protoplast, somatic and gametophyte triticale and wheat cells is concentration dependent and non saturable, enhanced at low temperature (4°C), and receptor independent. The permeation properties of CPP change upon complexing with macromolecules and according to recipient cell, as a result, cargo is internalised by an altogether different mechanism(s), either through endocytosis or macropinocytosis. Being single celled and without cell wall, protoplasts offer a comparable system to mammalian cell lines. Plant cell wall surrounding the cell membrane poses challenges in the uptake of cell-penetrating peptides, but can be overcome with permeabilisation pretreatment of targeted cells and tissues. The distinct ability of CPPs to deliver macromolecules that are otherwise restricted to cross the membrane has lead to development of novel peptide-mediated gene and protein delivery methods in somatic and gametophytic plant cells. Synthetic or in vivo produced nucleic acid, proteins and CPPs are blocks that conjugate to form nano-complexes in a relatively predictable manner. At the cross road of plant cell culture and nanocarrier technology CPP-mediated molecule delivery in plants can give rise to an entirely new field of 'phyto-nanobiotechnology'!

Amino acids in root exudates of *Miscanthus* × *Giganteus*

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Miscanthus × *giganteus* Greef et Deu is a perennial, rhizomatous C_4-grass originating from Southeast Asia. This species is sterile and probably natural hybrid involving *M.* × *sacchariflorus* (diploid) and *M. sinensis* (tetraploid) with a triploid chromosome, impossible of producing seeds. *M.* x *giganteus* belongs to group of *Miscanthus* species cultivated across Europe as a potential biofuels and seems to be promising plant for phytoremediations of heavy metals and organic pollutants. Phytoremediation of soil organic pollutants is based on their degradation by the soil microorganisms via direct catabolism or cometabolism, when supported by the water-soluble plant root exudates; knowledge about composition of water-soluble root exudates can be used for preparation of synthetic root exudates to be applied to soil to create artificial rhizosphere and simulate degradation of the organic pollutants. We have conducted to perform experiments on >15 years cultivation of *M.* × *Giganteus* in the autumnal period of growth to determine composition of the root exudates, which were collected for period of 2 h in demineralized water. Of 17 measured amino acids (aspartic acid, glutamic acid, serine, histidine, glycine, threonine, alanine, tyrosine, valine, methionine, phenylalanine, isoleucine, leucine, proline, arginine, cystine and lysine), aspartic acid, arginine, alanine and glutamic acid were the most abundant.

Expression of aspartic acid pathway genes in rice developing seeds (*Oriza sativa* L.)

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The major plant sources of protein are cereal and legume seeds. Rice is the staple food of half of the world population. Essential amino acids such as lysine, threonine, methionine and isoleucine are synthesized in higher plants in the aspartic acid biosynthetic pathway. Due to the low lysine and threonine concentrations in cereal seeds and their importance as essential amino acids, studies have been carried out in order to obtain information about the regulation of the pathway. To better understand some of the key enzymes controlling lysine and threonine metabolism in the rice endosperm during development, we performed relative quantification gene expression of *AK, HSDH, DHDPS, LL-DAP-AT, LOR-SDH,* and *TS* genes. Tubulin was selected as the reference gene. Rice seeds of IAC-165 cultivar were tagged during grain filling and collected into liquid nitrogen at stages 1, 2 and 3. Total RNA extraction was performed using Trizol reagent based on the single-step RNA isolation. The cDNA amplification was carried out according to Invitrogen SUPERSCRIPT^{TM} Kit. The expression *AK* gene reached its peak level at stage 3 while *HSDH, DHDPS, LL-DAP-AT e TS* are already declining in the same developmental stage. *HSDH* and *DHDPS* genes were more expressed and both reach its peak at stage 2. The highest expression pattern of *LOR-SDH* gene was observed at stage 2. *LOR-SDH* gene expression exhibited an higher level than the genes encoding the enzymes controlling lysine biosynthesis indicating a higher rate of lysine degradation which may explain at least in part the higher lysine content in rice.
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Investigate the magnitude of differences in total metabolizable protein among barley varieties

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Recently new genotypes of barley have been developed by Crop Development Center. However, no quantitative evaluation of protein supply to dairy cow has been done in terms of potential protein

degradation balance and total metabolizable protein (MP) supply. The objective of this experiment was to determine the magnitude of difference in total metabolizable protein (MP) supply of five feed type barley cultivars in comparison to Canada's most widely grown malting cultivar AC Metcalfe. Six, two row cultivars of spring sown barley, included AC Metcalfe CDC Cowboy, CDC Dolly, CDC Helgason, CDC Trey and McLeod were grown in the research field of University of Saskatchewan, Saskatoon, SK, Canada for three consecutive years commencing in 2003. The quantitative predictions were made in terms of: (1) Rumen synthesized microbial protein truly absorbed in the small intestine (AMCP); (2) Rumen undegraded feed protein truly absorbed in the small intestine (ARUP); (3) endogenous protein in the digestive tract (AECP); (4) total metabolizable protein supply in the small intestine (MP). The results showed that barley variety differed ($P < 0.05$) in AMCP ranging from 33.9 to 40.3 g/kg DM andAECP, but had no difference ($P > 0.05$) in ARUP with average of 47.5 g/kg DM. Total metabolizable protein ranged ($P < 0.05$) from 85.4 to 92.3 g/kg DM. In conclusion, barley variety affected total predicted MP supply.

Analysis of free amino acids in plants exudates

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Amino acids are ubiquitous and essential components in all living organisms. In this study, sunflower and maize plants were chosen as model plants for determination of aminoacids in phloem and xylem. Six weeks old plants were treated with cadmium(II) ions (0, 100, 500, 1,000 and 2,000 μM). Taking of a sample was carried out by rupturing of plants tissues and subsequent accumulation of exudate into filtration paper. The obtained samples were centrifuged (30 min, 16,000 rpm, 4°C). In both variants of plants cultivated at conditions mentioned above, level of glucose, which decreased according to cadmium(II) ions concentration from 50 to 20 μM, was spectrometric detected. Content of free amino acids was determined after their separation by using ion exchange chromatography with post-column derivatization by ninhydrin. The obtained calibration curves for Asp, Thre, Ser, Glu, Pro, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, His, Lys, and Arg were strictly linear ($R^2 = 0.999$, RSD = 4.5%). Limits of detection of individual amino acids were down to submicromolar concentrations. In plant exudates, contents of amino acids threonine, glycine, alanine, tyrosine, histidine, lysine, and arginine were particularly determined. In the event of the plants stress, the increasing of lysine and phenylalanine content for more than 100% in comparison with control group of plants was evident.

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Polyamines

Determination of Global effect of polyamines on regulating growth related gene expression

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Our present study aims at exploring the molecular mechanisms that underlie the involvement of polyamines in regulating cellular proliferation. We decided to undertake a genome wide approach by testing global changes in pattern of gene expression occurring in polyamine-depleted cells. RNA was isolated from NIH3T3 cells treated with DFMO for 4 days. Since FACS analysis revealed that DFMO treated NIH3T3 cells arrest predominantly at the G1 phase, G1 cells were isolated by cell sorting and compared to G1 cells from growing culture. DMSO treated cells that also arrest predominantly at the G1 phase were used as a control. The RNA was used for preparing probes that were hybridized to affymetrix DNA arrays. Clear differences were observed between the DFMO arrested, growing cells and the DMSO arrested cells. DNA array analysis was also used to determine the temporal sequence of changes in gene expression occurring up to the point of complete arrest and after the arrest was relieved by the addition of exogenous polyamines. Since there are claims that polyamines affect cellular proliferation predominantly through eIF5A hypusination, we used DNA array analysis to compare the pattern of changes in gene expression in cells that were growth arrested by DFMO versus GC7 (hypusination inhibitor) treatments.

We also revisited the important question of whether antizyme affects cellular proliferation solely by affecting proteins that regulate cellular polyamine metabolism or whether antizyme regulates other cell cycle regulators.

Phase II prospective trial associating a polyamine free diet and docetaxel in hormone refractory prostate cancer patients: preliminary results

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Introduction: Polyamine free diet (PFD) has anticancer properties and synergistic effects with chemotherapy in pre-clinical models. We are assessing quality of life of HRPC patients treated by chemotherapy with PFD.
Methods: 12 patients with metastatic HRPC were enrolled in a phase II trial associating a PFD with docetaxel (75 mg/m^2, 6 cycles every 3 weeks). PFD is an industrially manufactured canned liquid nutrient covering all nutritional needs, proposed during the first two weeks. A polyamine reduced meal is then introduced for 2 weeks, then two meals a day with one PFD meal for the rest of the trial. PFD was started 3 weeks before docetaxel induction. Quality of life was assessed by EORTC QLQC-30 questionnaires, performance status by the WHO scale and pain by pain scales and analgesic consumption. Body weight and blood parameters were regularly evaluated.
Results: 3 patients did not complete the protocol. All patients are evaluable for the PFD alone period and nine patients for the PFD-docetaxel association.

Quality of life, performance status and pain were significantly improved during the protocol, particularly during the PFD alone period, with no significant clinical or biological side effects usually observed with docetaxel. PSA reduction is observed in six patients. **Conclusion:** these preliminary results show a significant quality of life improvement with PFD alone or with docetaxel in HRPC.

Pleiotropic effects of manipulating putrescine metabolism on the cellular transcriptome and the metabolome

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The polyamine metabolic pathway is intricately connected to the metabolism of several amino acids. While ornithine and arginine serve as direct precursors of putrescine, they themselves are synthesized from glutamate in multi-step processes involving several enzymes. Additionally, glutamate is an amino group donor for the synthesis of several other amino acids, and acts as a substrate for the biosynthesis of proline and γ-aminobutyric acid, metabolites that play crucial roles in plant stress responses. Poplar (*Populus nigra* × *maximowiczii*) cells, transformed with a constitutively expressing mouse ornithine decarboxylase gene were used to study the effect of up-regulation of putrescine biosynthesis (and concomitantly its enhanced catabolism) on cellular contents of all protein amino acids, two non-protein amino acids (ornithine and γ-aminobutyric acid), and the general metabolome. Up-regulation of putrescine metabolism affected the accumulation of most amino acids in the cells. There was a decrease in cellular glutamine, glutamate, ornithine, arginine, histidine, serine, glycine, phenylalanine, tryptophan, aspartate, lysine, leucine, and methionine; the contents of alanine, threonine, isoleucine and γ-aminobutyric acid increased. An overall increase in total cellular nitrogen and carbon was also observed in high putrescine metabolizing cells. In the same two cell lines, we also found that upregulation of putrescine biosynthesis had effects that went beyond the metabolism of amino acids, particularly in the organic acids of the glycolytic pathway and the TCA cycle. Thus genetic manipulation of the polyamine pathway showed pleiotropic effects covering the entire cellular metabolism.

Deciphering functions of poly-plural polyamines via novel transgenic plant systems

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Transgenic tomato plants homozygous with the introduced gene construct ySAMdc-E8 were developed in order to understand the role of higher polyamines, spermidine (Spd) and spermine (Spm), in plant biology. The NMR-based metabolite profiling suggests that Spd and Spm are perceived as 'signaling' organic-N metabolites by the fruit cells, revive metabolic memory and stimulate carbon sequestration, enhanced synthesis of biomolecules, and nitrogen use efficiency. Transcriptome analysis revealed a large number of differentially expressed genes, representing discrete functional categories as well as novel pathways: amino acid biosynthesis, carotenoid biosynthesis, cell wall metabolism, chaperone family, flavonoid biosynthesis, fruit ripening, isoprenoid biosynthesis, polyamine biosynthesis, signal transduction, stress/defense related, transcription, translation, and vacuolar function. Our analyses suggest that polyamines act as anti-apoptotic regulatory molecules and cross talk with specific signaling pathways. We have also identified gene clusters in tomato genome which are targets of polyamine action and reveal complex nature of Spd and Spm action in regulating plant processes.

Agmatine and brain mitochondria: transport mechanism and prevention against permeability transition induction by Ca^{2+}

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Agmatine is a biogenic amine synthesized by arginine decarboxylase (ADC). At physiological pH it is completely protonated and behaves as a divalent cation. Agmatine is metabolized by two distinct enzymes: (1) a diamine oxidase in peripheral tissues, which catabolizes agmatine to guanidobutyraldehyde and hydrogen peroxide, (2) agmatinase, which catalyzes the formation of urea and putrescine in brain. Agmatine is generally transported across the plasma membrane as a monocation and the transporter has been identified as the extraneuronal monoamine transporter (EMT) or the organic cation transporter 2 (OCT-2). In brain agmatine exhibits neurotransmitter and neuromodulator properties, is released by membrane depolarization, interacts with receptors (i.e. α-adrenergic, imidazoline).

Previous papers demonstrated that agmatine exhibits strict relationships with brain mitochondria. In fact, its presence has been recognized in these organelles as well as that of metabolic enzymes, ADC and agmatinase. Also the I_2 receptor, which binds agmatine, is located in mitochondrial membrane. Taking into account these observations, the aim of this study is to evaluate if agmatine is transported in brain mitochondria and what is the physiological role of this process.

Results show that agmatine is taken up by an electrophoretic mechanism showing different as regards previously discovered in liver mitochondria. Indeed the interaction between agmatine and brain mitochondria evidence a clear protective dose-dependent effect, by the diamine, against the permeability transition induction by supraphysiological Ca^{2+}concentration. This suggests an involvement of agmatine in modulating the triggering of the pro-apoptotic pathway.

Polyamine reduced diet and low dose metronomic cyclophosphamide in hormone refractory prostate cancer (HRPC) patients

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Introduction: Reducing exogenous dietary polyamines may favourably impact quality of life in HRPC patients. In pre-clinical

models, polyamine deprivation potentializes low dose chemotherapies. We have assessed the association of a polyamine reduced diet with low dose metronomic cyclophosphamide chemotherapy in HRPC patients.

Methods: 22 patients, mean age 75 ± 8 (56–87) years with HRPC had a polyamine reduced diet as previously described and low dose metronomic cyclophosphamide, 100 mg daily, 3 weeks out of four, continued until PSA progression. WHO performance status, EORTC pain scale, body weight, serum prostate specific antigen (PSA) and blood counts were assessed.

Results: WHO performance status, body weight, haemoglobin and platelets were not impaired. For white blood cells, only asymptomatic grade 2 lymphopenia was regularly observed. No significant toxicity was observed for patients ≥75 years (64%). PSA reduction or stabilization was observed for 16 patients (72%) for a mean 6 ± 4 months. Six patients (33%) had a ≥50% PSA decrease.

Conclusions: polyamine reduced diet and low dose metronomic cyclophosphamide is well tolerated with little toxicity, particularly in the elderly patient. A ≥50% PSA reduction response can be observed for a mean six months, which is appreciable in this aggressive disease.

Novel convenient synthesis of biologically active esters of hydroxylamine

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Substrate-like *O*-substituted hydroxylamines are effective inhibitors of carbonyl-dependent enzymes of amino acids metabolism, including ornithine- and *S*-adenosylmethionine decarboxylases. Other biochemical applications of *O*-substituted hydroxylamines cover the preparation of different conjugates of peptides, nucleic acids, and recently the use of aminooxy resins for the isolation of carbohydrates. All above requires the preparation of rather complicated functionally substituted derivatives of hydroxylamine. Most of published synthetic methods start with corresponding alkyl halides, which react with *N*-protected hydroxylamine derivative. Subsequent transformations in the radical of the intermediate ester and removal of *N*-protection group result in required *O*-substituted hydroxylamine derivatives. A family of functionally substituted alcohols is much diversed, as compared with corresponding alkyl halides. However, the only preparative method to obtain *O*-substituted hydroxylamines starting from alcohols is Mitsunobu reaction. Unfortunately, the properties of *N*-alkoxyphtalimide group make impossible to perform many of commonly used reactions of nucleophilic substitution to introduce functional group(s) in the side-chain of hydroxylamine ester. To overcome this serious disadvantage we suggest using ethyl *N*-hydroxyacetimidate, which is easily alkylated with readily available mesylates of functionally substituted alcohols in a high yield. This opens a possibility to perform wide spectra of the transformations in the side chain of *N*-protected ester of hydroxylamine and to obtain required functionally substituted structures. A set of examples illustrate the syntheses of hydroxylamine-containing inhibitors of ornithine decarboxylase and also synthesies of 4-methylagmatine—earlier unknown mimetic of agmatine.

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Influence of different abiotic stresses on the accumulation of polyamines

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Environmental stresses influence the growth, development and agricultural production of cereals adversely. Many plants are able to increase their stress tolerance in response to destructive effects. This process contains many chromosome affected biochemical and physiological changes, for example in accumulation of compatible solutes such as carbohydrates, free amino acids and biogenic amines.

Biogenic amines are nitrogen-containing low molecular weight compounds with biological activity. They can be formed and catabolised during the normal metabolism of plants, animals and microorganisms. Biogenic amines are derived mainly from decarboxylation of amino acids through substrate-specific decarboxylase enzymes. In plants, polyamines (spermidine, spermine and their precursor putrescine) are the most common biological active amines. They are involved in various physiological processes such as development, senescence and stress responses. During stress, polyamines especially the diamine putrescine, act as reactive oxygen species scavenging, protect membranes and macromolecules and play a role as signalling molecules in expression of several stress-related genes.

The aim of our work to investigate the effect of different abiotic stresses (low temperature, osmotic stress) and chromosome 5A of wheat, one of the main regulators of stress tolerance, on the accumulation of biogenic amines. The moderately frost-sensitive *Triticum aestivum* cv. Chinese Spring wheat cultivar and the frost-tolerant Chinese Spring (Cheyenne 5A) and the frost-sensitive Chinese Spring (T. spelta 5A) chromosome 5A substitution lines were used in the experiments. Significant differences were found between the sensitive and the tolerant genotypes. Quantitative determination of polyamines was accomplished by ion-exchange chromatography (amino acid analyzer).

The change of antizyme inhibitor expression and its possible role during mammalian cell cycle

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Antizyme inhibitor (AIn), a homolog of ODC, binds to antizyme and inactivates it. We report here that AIn increased at the G1 phase of the cell cycle, preceding the peak of ODC activity in HTC cells in culture. During interphase AIn was present mainly in the cytoplasm and turned over rapidly with the half life of 10–20 min, while antizyme was localized in the nucleus. The level of AIn increased again at the G2/M phase along with ODC, and the rate of turn-over of AIn in mitotic cells decreased with the half-life of approximately 40 min. AIn was colocalized with antizyme at centrosomes during the period from prophase through late anaphase and at the midzone/midbody

during telophase. Thereafter, AIn and antizyme were separated and present at different regions on the midbody at late telophase. AIn disappeared at late cytokinesis, whereas antizyme remained at the cytokinesis remnant. Reduction of AIn by RNA interference caused the increase in the number of binucleated cells in HTC cells in culture. These findings suggested that AIn contributed to a rapid increase in ODC at the G1 phase and also played a role in facilitating cells to complete mitosis during the cell cycle.

Effect of taurine on oxidative stress and pao activity in mitochondria isolated from the testes of rats with alloxan-induced diabetes mellitus

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Although polyamines are present in high concentrations in reproductive tissues, their roles still remain to be elucidated. Polyamino oxidase (PAO) catalyzes the oxidation of spermine, spermidine and their acetylated derivatives. Degradation products, such as H_2O_2 and aldehyde(s), leads to DNA fragmentation and cell death by apoptosis. Recently, it has been found that taurine may enhance sperm production and motility. There is little evidence to suggest that endocrinopathy or spermatogenic disorders are the primary cause of infertility in diabetics. The study aimed to examine the mechanisms by which taurine exerts beneficial effects on oxidative stress and polyamine catabolism in isolated testes mitochondria of aloxan diabetic rats. A single dose of aloxane (170 mg/kg BW) significantly increased testes PAO activity (4.19 ± 0.33 vs. control 3.4 ± 0.21; $P < 0.01$) as well as MDA level (10.8 ± 0.93 vs. control 6.0 ± 0.42; $P < 0.001$). Taurine (1.6 mmol/kg BW), given intraperitoneally 1 day before aloxane, as well as continually as single daily dose for 5 days, significantly decreased testes PAO activity (2.83 ± 0.72; $P < 0.001$) and concentration of MDA (8.2 ± 0.94; $P < 0.005$). Application of taurine to control animals produces the same effects. Obtained in vitro results indicate that taurine had no effect on MDA in a dose-dependent manner in the model of Fe^{2+} induced oxidative stress in the suspension of phospholipid liposomes. We propose that taurine has a restorative effect on aloxan-induced testes damage by decreasing oxidative stress and PAO activity, with potential therapeutic value in diabetic infertility.

Comparative study of antiproliferative and antitumor properties for novel heterocyclic compounds mediated by polyamines' metabolism in tissues with rapid cell proliferation

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Influence of new synthetic heterocyclic compounds on polyamine metabolism in tissues with rapid cell proliferation was investigated. Quantitative assessment of action of these substances on synthesis and oxidative desamination rate of putrescine, spermidine and spermine and on the levels of polyamines in test-systems was carried out. Cell-free testing systems of regenerating liver and hepatoma Γ-27 were used. Tissue-culture of tumor cells (transformed fibroblasts of hamster—L-cells, human ovarian carcinoma—CaOv, melanoma of mice—B16-F10) were used as model cell testing systems.

Obtained results allows to suppose presence of *carcinogenic* properties in dioxaboreninopyridine and benzimidazole derivatives, *proliferative* properties in piperidone derivatives, *antiproliferative* properties in aniline derivatives, *oncoprotective* properties in bis-uridile derivatives and properties of inductors of cytodifferentiation in azafluorene and xantine derivatives.

Perspective of further investigation of azafluorene, xantine and Bis(uracilyl) PA analogs as potential anticancer drugs was shown. It was established advantage of effectiveness of azafluorene derivatives over other heterocyclic substances.

Regulation of polyamines' metabolism by benzimidazole and azafluorene derivatives in tissues with rapid cell proliferation

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Polyamine (PA) metabolism can be a suitable experimental target in studying the proliferative activity of tissues and the biological properties of substances that influence that activity.

The biochemical properties of 12 derivatives of benzimidazole (1,2) and azafluorene (3-12) were studied: 1)7-aminopyrido [1,2-α]benzimidazole, 2)7-nitropyrido [1,2-α] benzimidazole, 3) 1-amino-4-azafluoren, 4)1-amino-4- azafluorenol, 5)9-dicyanomethylene-4- azafluoren, 6)9-[α-(β-hydroxyethyl)aminomethylene]-4-azafluoren, 7)9-[α-pyridilaminomethylene]-4-azafluoren, 8)1-amino-9-phenylamino-4- azafluorene, 9)1-amino-4-azafluorenon-9, 10)1, 4-diazoacetonaphthylene[1.2-f]-fluorantene, 11)2-methoxycarbonyl-(β-benzoilethyl)aniline, 12)5-(2-methoxycarbonyl)-phenyl-α-furfural.

The effect of substances on PA disintegration, levels and synthesis has been quantitatively assessed on a model cell-free testing system of tissue with rapid cell proliferation: the rat regenerating liver and hepatoma G-27. The carcinogenic and antitumour properties of new chemical substance have been assessed by character and level of their effect on the rate of PA disintegration and synthesis during 1 h incubation.

All tested substances, except (1), notably increase spermidine disintegration. All studied chemical compounds decrease ornitinedecarboxylase (ODC) activity in regenerating liver. Substances (3), (8), (9) and (10) appeared to be the most effective inhibitors of putrescine and PA synthesis rate. The same compounds (3, 8, 9, 10) decreased PA levels in the most active way.

Benzimidazole derivatives 1 and 2 decrease in 1,5-1,7 times aminoxidase activity in regenerative liver so they are potentially carcinogenic agents. Azafluorene derivatives 8 and 10 decreased the level of polyamines 50–70% less**.** Therefore, these substances could suppress carcinogenic proliferation. Compounds 3, 8, 10, decreasing ODC activity 30–50% less, may be potential antiproliferative agents. These testing system is very suitable in primary collection of low-toxic substances with a potential antiproliferative activity and in forecasting carcinogenic properties of chemical compounds.

Study of immunogenic properties of the antileukemic substance L-lysine-α-oxidase from *trichoderma harzianum rifai*

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L-lysine-α-oxidase is a new enzyme obtained from fungi of the genus *Trichoderma*. The enzyme catalyzes oxidative deaminization of essential amino acid L-lisyne.

Study of immunogenic properties of L-lysine-α-oxidase was carried out with repeated injection of the preparate to mice (C57B1 line). Substance of L-lysine-α-oxidase was injected intravenously in therapeutic doses 35 E/kg daily during 5 days. Blood was tested weekly during four weeks from the immunization start of the group of seven animals. Obtained blood serums were analyzed by immune-enzyme analysis.

The dynamics of humoral immune response to the introduction of the enzyme L-lysine-α-oxidase did not differ from the characteristics of antibody-formation in response to the introduction of protein antigens. The maximum content of antibodies in animals was from the seventh until fourteenth day of the experiment. Antibody titer decrease was observed during the next days.

It should be noted that the titers of immune-enzyme analysis are very low. This fact indicates a low immunogenicity of enzyme preparation in tested dose of 35 E/kg or 0.8 mg protein/kg. We can compare these results with immunogenicity of the well-known anticancer drug L-asparaginase from *E. coli*, allowed for clinical application. The introduction of L-asparaginase five times in a dose 300 E/kg (2.0 mg/kg) leads to an increase of antibody titer to 1/256, which considerably exceeds titers to L-lysine-α-oxidase (1/64).

Thus, the intensity of immune response to L-lysine-α-oxidase does not exceed or even slightly lower than the other enzyme antitumor drug L-asparaginase.

Kinetic characterization of a novel copper amine oxidase activity from rat liver mitochondria matrix

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The present study reports preliminary results on the presence of a novel Copper containing amine oxidases (Cu-AO, EC 1.4.3.6.) in rat liver mitochondria lysates. Such enzymatic activity was found in the soluble mitochondrial fraction, obtained by simple osmotic shock. The mitochondrial amine oxidase was isolated by affinity chromatography on a newly synthesised spermine-Sepharose. SDS-PAGE showed a single band at about 60 kDa. Upon chromatographic purification, the enzymatic activity was very labile. The crude enzyme activity was tested by spectrophotometric measurements, determining hydrogen peroxide production following oxidative deamination of different substrates, such as polyamines (spermine, spermidine, putrescine and cadaverine) and monoamines (dopamine and benzylamine). The activity, observed on polyamines and not on monoamines, was inhibited by semicarbazide and azide, but not by pargyline, clorgyline and L-deprenil. Enzyme specificity was tested on several diamines characterized by different carbon atom chain length in the range 2–6 carbon atoms. The highest activity was found with 1,2-diamino-ethane and the highest affinity with 1,5-diamino-pentane. The above reported results suggest the presence of a novel copper-dependent amine oxidase in liver mitochondria matrix.

The effect of hypusine modification and acetylation on the intracellular localization of eIF5A

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Eukaryotic translation initiation factor 5A (eIF5A) is a highly conserved translation factor essential for eukaryotic cell proliferation. This is the only cellular protein that contains hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine] which is formed post-translationally by conjugation of a portion of the polyamine spermidine to a specific lysine residue. Hypusine synthesis occurs by way of two consecutive enzymatic reactions catalyzed by deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH). eIF5A also undergoes an acetylation at specific Lys residue(s) (Lys47). Although eIF5A has been reported to be distributed in cytoplasm was well as in nuclei, functional significance of nuclear eIF5A is unknown. In this study, we have investigated the effect of hypusine modification and acetylation on subcellular localization of eIF5A using non-tagged, Flag-tagged and GFP-tagged eIF5A. Immunocytochemical analyses showed differences in intracellular distribution of unmodified eIF5A precursor, eIF5A(Lys) and the hypusine-containing eIF5A. Whereas unmodified eIF5A(Lys) is more concentrated in nuclei than in cytoplasm, the hypusine form is localized mainly in cytoplasm. eIF5A mutant proteins that is defective in hypusine modification (K50A, K50R) were localized similar to eIF5A(Lys), whereas the hypusine-modified mutant proteins (K47A, K47R and K68A) were localized preferentially in cytoplasm. Although acetylated eIF5A was mainly found in cytoplasm, there was little difference in the overall eIF5A distribution pattern between the acetylatable and non-acetylatable eIF5A mutants, presumably because only a small fraction of eIF5A was acetylated. These findings demonstrate that the hypusine modification of eIF5A maintains the active eIF5A(Hpu) in the cytoplasmic compartment, where it is required for protein synthesis.

Deoxyhypusine hydroxylase from plasmodium: a protein with E–Z type heat repeat motifs present in photosynthetic phycocyanin lyase of cyanobacteria

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One of the most important issues facing global health today is the need for new, effective and affordable drugs against malaria which are limited by factors ranging from parasite resistance to safety and cost.

In search for new targets for antimalarials we have investigated the biosynthesis of hypusine present in eukaryotic initiation factor (eIF-5A), in particular the deoxyhypusine synthase (DHS), and eIF-5A from the different human malaria parasites *P. falciparum* and *P. vivax*. We now describe the cloning and expression of deoxyhypusine hydroxylase (DOHH), the enzyme that completes the modification of eIF-5A through hydroxylation. The sequence of the *dohh* cDNA revealed an open reading frame of 1,236 bp encoding a protein of 412 amino acids with a calculated molecular mass of 46.45 kDa and an isoelectric point of 4.96. Interestingly, DOHH from *Plasmodium* has a FASTA SCORE of only 27 compared to its human ortholog and contains several matches similar to E–Z type HEAT-like repeat proteins (IPR004155) (InterPro), PF03130 (Pfam), SM00567 (SMART) present in phycocyanin lyase subunits of cyanobacteria. Expression of DOHH in *E. coli* resulted in a signal of 42 kDa in SDS PAGE analysis. The purified protein displayed hydroxylase activity in a novel *in vitro* DOHH assay. In contrast, phycocyanin lyase activity was absent.

The *dohh* gene is present as a single copy gene in the genome of *Plasmodium* and is transcribed in both developmental stages. The N-terminus of *Plasmodium* DOHH contains a signal peptide which might direct the protein to a different cellular compartment.

Biogenic polyamines: the structural variable

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Biogenic polyamines—putrescine (PUT, $H_2N(CH_2)_4NH_2$), spermidine (SPD, $H_2N(CH_2)_3NH(CH_2)_4NH_2$), spermine (SPM, $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) or agmatine (AGM, NH_2-CH_2-CH_2-CH_2-CH_2-NH-C(-NH_2)(= NH))—are ubiquitous in cells of higher organisms and fundamental characters in processes essential to life as cell growth and differentiation, through tightly regulated concentration-dependent routes. MGBG (methylglyoxal *bis*(guanylhydrazone)), for instance, is a competitive inhibitor of *S*-adenosyl-L-methionine decarboxylase, therefore involved in the biosynthesis of SPD and SPM.

The activity of a biologically relevant compound, however, does not rely solely on its chemical properties, but it is also dependent on its conformational preferences, that must be accurately determined for a thorough understanding of its function and mechanisms of action.

The present report describes the conformational analysis of PUT, SPD, SPM and AGM, as well as of MGBG, through quantum mechanical calculations coupled to vibrational spectroscopy techniques (infrared, Raman and Inelastic Neutron Scattering).

The conformational preferences of this kind of alkylamines, comprising different protonation sites, are ruled by their biochemical environment (i.e. pH, type of tissue and nearest biomolecules or receptors). They rely on a balance between the formation of stabilising intramolecular (N)H: N and/or (C)H: N hydrogen-bonds, and the minimisation of steric and electrostatic repulsive interactions. Under physiological conditions, polyamines occur are either totally protonated, natural polycations, or as partially ionised species (in particular situations).

The establishment of these highly sensitive structure-activity relationships allows to achieve a better understanding of polyamines' particular biological roles, and to predict mechanisms for their transport and mode of action.

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Delivering anti-cancer agents to human leukaemic cells via polyamine transport system

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One of the big challenges to oncology is to deliver cytotoxic agents selectively to cancer cells. Currently, the lack of selectivity has limited the clinical use of many of the available drugs. By enhancing specific cell targeting, non-specific toxicities can be minimised. By taking advantage of the highly active polyamine uptake system (PTS) in tumour cells we have developed a novel way to deliver polyamine–drug conjugates selectively to cancer cells. The PTS is relatively non specific and does not differentiate between natural polyamines and structural analogues and so can accommodate a range of polyamine conjugates. Using this delivery system, chemotherapeutic drugs attached to a polyamine chain can be targeted to tumour cells via selective uptake and at the same time the toxic effects on normal cells can be decreased. A series of novel compounds, which include a DNA intercalator, anthracene, with a polyamine side chain have been synthesized.

The cytotoxicity of two compounds, namely 9-anthracenylmethyl-butanediamine (Ant 4), N^1-anthracenylmethyl-4,4-triamine (Ant 4,4), was investigated as a paradigm for the delivery of cytotoxic agents via PTS using human leukaemic cells (HL-60) as in vitro model. The compounds showed significant toxicity and apoptosis. An interesting finding was the significant polyamine depletion caused by treatment with both compounds after 48 h exposure. Indirect methods were used to show that the conjugates used the PTS.

In summary, the preliminary data indicate the Ant 4 and Ant 44 recognise and utilise the PTS. This means of drug delivery via PTS represents a promising approach for therapies in cancer.

Prognostic value of erythrocyte polyamine levels in renal cell carcinoma patients. A prospective analysis in 418 cases

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Introduction: Erythrocyte polyamines (EPA) Spermidine (Spm) and Spermine (Spm), are of prognostic interest in prostate carcinoma. We have assessed the value of EPA as prognostic markers in renal cell carcinoma patients.

Methods: Blood samples for EPA determination by HPLC were collected in 418 patients before nephrectomy. Results are expressed in nmol/8.10^9 erythrocytes. TNM stage, Fuhrman grade, ECOG performance and survival statuses were assessed.
Results: Median age at diagnosis was 64 (21–88) years. Median follow-up was 41 (1–214) months. Median Spm and Spd levels were 4.7 (1–83) and 9 (2–86) nmol/8.10^9 erythrocytes, respectively. Spm and Spd were associated with T stage ($P = 0.0001$), and ECOG ($P = 0.0001$). Spm was associated with Fuhrman grade ($P = 0.0001$) but not with N and M stages. In univariate analysis, tumour size, TNM stage, Fuhrman grade, Spm and Spd ($P < 0.0001$) were predictors for cancer specific survival. In multivariate analysis: TNM stage, ECOG and Spd remained independent prognostic factors ($P = 0.0001$). When Spm and Spd were dichotomized in qualitative variables they were both retained as independent predictors.
Conclusions: EPA determination seems to be a promising new prognostic tool in renal cell carcinoma.

Chloroquine potentiates cytotoxicity induced by enzymatic spermine metabolites on human cancer cells

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Chloroquine (CQ) is a derivative of quinine and has been widely used as an anti-malarial and anti-inflammatory drug. Many investigations on the causes of the limited success of anticancer chemotherapy have oriented on cellular and molecular mechanisms underlying the multidrug-resistance phenomenon. Several studies have been performed to overcome the MDR phenotype and to develop innovative strategies effective against multidrug resistant (MDR) cancer cells. We are exploring the cytotoxic effect on both wild-type (WT) and MDR colon adenocarcinoma (LoVo) and melanoma (M14) cells induced by CQ, administered alone or in association with bovine serum amine oxidase (BSAO) and spermine.

When the cells were pre-treated with CQ ≤ 50 μM, kept in incubator at 37°C for 24-48 h and then treated with spermine/BSAO ≤ 6 μM, the clonogenic assay showed that CQ was able to sensitize both WT and MDR cells, either M14 or LoVo, to the spermine metabolites. It was observed greater cytotoxicity on cells pre-treated with CQ than on those treated with only BSAO/spermine. Transmission electron microscopy observations showed that the pre-treatment of the cells with CQ caused the formation of numerous cytoplasmic vacuoles. The pre-treatment also increased the number of lysosomal structures as shown by confocal microscopy, indicating a contribution of the lysosomotropic properties of CQ to the sensitization of the tumor cells to BSAO/spermine metabolites. This study indicates that CQ, in association with BSAO/spermine, could potentiate the effects of the enzymatic oxidation products of spermine and might be a new approach in anti-neoplastic therapy, particularly against MDR cancer cells.

Integration of metabolic information: developing new tools and hypothesis to study amino acid/amine metabolism regulation

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Systems Biology can make possible to approach the characterization of new emergent properties that cannot be discovered from reductionistic studies. In Systems Biology it is becoming a routine task to build models of increasing complexity on a given biochemical network or pathway of interest. In order to help this task, we developed SBMM Assistant, a tool built using an ontology-based mediator, and designed to facilitate metabolic modeling through the integration of data from repositories that contain valuable metabolic information. SBMM Assistant (freely available for academic use at http://www.sbmm.uma.es) is an SBML-compatible and user-friendly tool that gives the user the ability to capture, enrich, generate and visualize biological networks, which was designed as an assistant for kinetic modeling. In this communication, we present several applications of SBMM Assistant to develop new tools and facilities to predict (environmental and/or genetic) alterations in cationic and sulphur amino acids related to different rare diseases and nutritional alterations. Metabolic modeling-driven hypothesis are validated and enriched through experimental molecular biology approaches including proteomic studies. By using this systemic approach, some metabolic effects were observed that, otherwise, they would have been hard to be detected and explained from a reductionistic view. The strategy and its results will be shown and discussed.
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Structural studies on human *S*-adenosylmethionine decarboxylase

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Decarboxylated *S*-adenosylmethionine (AdoMet) provides an aminopropyl group for the biosynthesis of spermidine from putrescine, or of spermine from spermidine. *S*-Adenosylmethionine decarboxylase (AdoMetDC) is a pyruvoyl-dependent enzyme in which the pyruvoyl cofactor is generated by autoserinolysis of a proenzyme. Both the autoprocessing reaction and the decarboxylation reaction for human AdoMetDC are activated by putrescine. In addition, human AdoMetDC is highly selective for ligands that contain a positive charge corresponding to the sulfonium center of AdoMet. We have used site-directed mutagenesis, biochemical studies, theoretical calculations and X-ray crystallography to investigate the basis for putrescine activation and substrate specificity. Comparisons of putrescine bound and putrescine free structures of human AdoMetDC show that the conformation of a loop containing residues 312–320 depends on the

presence or absence of putrescine. Furthermore, the putrescine molecule is linked to His243, an important active site residue nearly 20 Å away, by a hydrogen bonding network. The requirement for a sulfonium center, or equivalent positive charge, results from cation–π interactions involving Phe7 and Phe234, and from electrostatic interactions between the positive charge and the adenine N3 atom when the AdoMetDC is in the *syn* conformation. These observations have implications for the development of AdoMetDC inhibitors, which may be useful for anticancer or antiparasitic chemotherapy.

Antizyme is necessary for conversion of pancreatic tumor cells into glucagon-producing differentiated cells

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Human pancreatic tumor cell lines, AsPC-1, PANC-1, MIA paca2, KP-1 and KP-59 cells, can be induced to differentiate into pancreatic hormone-producing cells by brief trypsin treatment and the subsequent culture in a serum-free, chemically defined medium. During culture, AsPC-1 cells formed cell clusters resembling the pancreatic islets, expressed genes associated with the pancreatic development and produced glucagon but not insulin. When PANC-1, MIA paca2, KP-1 and KP-59 cells were treated and cultured the same way, they underwent similar morphological changes and produced insulin and glucagon. We used these systems to identify intracellular regulatory molecules involved in the conversion of pancreatic tumor cells into glucagon-producing cells. We found that the expression of antizyme 1, a negative regulator of ornithine decarboxylase, was increased and its localization was altered from the nucleus to the cytoplasm during AsPC-1 cell differentiation. Transient transfection of AsPC-1 cells with antizyme 1 siRNA resulted in inhibition of the morphological and functional cell differentiation as well as the specific suppression of antizyme 1 expression. In contrast, constitutive overexpression of antizyme 1 in AsPC-1 cells led to the enhancement of glucagon production. We also found that PANC-1 cells reduced the expression of glucagon mRNA when treated with antizyme 1 siRNA. These results suggested that antizyme 1 was necessary for conversion of pancreatic tumor cells into glucagon-producing cells. Glucagon production in AsPC-1 cells was not affected by addition of putrescine, suggesting that the polyamines were not directly involved in the antizyme 1-mediated conversion of pancreatic tumor cells to differentiated state.

Spermidine influence on arginase activity and nitric oxide synthesis relationship in different brain structures during experimentally induced seizures

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Introduction: Interactions of NO and polyamine metabolism, considering that they originate from the same substrate, L-arginine, and their CNS functions, is very intrigant area in neuropathology research. Thatswhy the aim of this study was to examine exogenous spermidine effects on NO production and arginase activity during seizures induced by penthylene tetrazole (PTZ).
Materials and methods: Male adult Wistar rats (300 ± 50 g), were used for the experiment. The animals were allocated into four experimental groups (8 in each): I (control)—treated by saline intraperitoneally (i.p.) applicated; II (PTZ)—seizures were induced by i.p. application of penthylenetetrazole (100 mg/kg bw); III (Spd)—the animals were treated by i.p. application of spermidine (1 mg/kg bw 50 min before PTZ); and IV (Mid)—treated by antiepileptic Midazolam in a dose of 100 mg/kg b.w. 45 min before PTZ. The animals were followed 4 minutes after the symptoms onset. In cortex, striatum, hyppocampus, cerebellum and brainstem NO_2 + NO_3 levels and arginase activity were determined.
Results: Spermidine, exerted proconvulsive effects related to seizures induced by PTZ, shortening the time of seizure symptoms onset. In all examined brain structures spermidine induced highly increased NO production related to values in PTZ group, confirming NO signalling system involvement in spermidine effects during seizures. Arginase acitivity was significantly diminished, suggesting its possible role in NO production regulation.
Discussion: The present study reveals that exogenous spermidine potentiates PTZ convulsant activity in rats. This effect is, at least partly, mediated by increased nitric oxide synthesis with the most pronounced effects in cortex and striatum.

Hydroxylamines for the regulation of the enzymes of polyamine metabolism

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Spermine and spermidine metabolizing enzymes belong to different families and respectively chemically very different compounds were used to regulate their activities. Among these inhibitors the derivatives of hydroxylamine occupy rather unique place due to specific properties of aminooxy- and hydroxyamino groups. Their ability to form stable oximes with carbonyl compounds fast and quantitatively, as well as decreased pKa values and decreased nucleophility of aminooxy group as compared with aliphatic amino group, made it possible to obtain effective or in some cases even very effective inhibitors of target enzymes. Hence, within the same class of chemical compounds it turned possible to obtain the regulators of practically all polyamine metabolic pathways. Some recent data, including an attempt to prepare actively-transported inhibitors will be discussed.
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Putrescine–pectin conjugate: synthesis and biotechnological use

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The biogenic diamine putrescine (1,4-diaminobutane) was covalently linked to low-methoxyl pectin, preliminarly oxidized with metaperiodate (Villalonga et al. in World J Microb Biotech 22:595, 2006), to produce a new aminated biodegradable polymer. The synthesized

putrescine-pectin conjugate (PPC) was found to be able to act as an effective acyl acceptor transglutaminase (TGase) (Mariniello and Porta in Prog Exp Tumor Res 38:174, 2005) substrate in vitro by using both dimethylated casein and soy flour proteins (SFP) as acyl donors. SDS-PAGE experiments, followed by fluorography of the gel, indicated that mono-dansylcadaverine, a well known amino donor TGase substrate, dose-dependently antagonized PPC covalent binding to the acyl donor proteins. Hence, PPC was tested to prepare, in combination with SFP, edible films in the absence or presence of purified microbial TGase (Mariniello et al. in J Biotech 102:191, 2003). Characterization of the films produced in the presence of the enzyme catalyzing PPC covalent binding to SFP showed a significant decreased water vapour permeability with respect to the ones obtained with non-aminated pectin, as well as improved mechanical properties, such as tensile strength and elongation to break. A possible use of PPC containing films as coatings for drug delivery is suggested.

Inducing in vitro and in vivo antiproliferation and differentiation by azafluorene derivatives

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Substances inhibiting an oxidative deamination of polyamines are likely to manifest carcinogenic properties. And vice versa, chemical compounds activating the process of oxidative disintegration of putrescine and PAs may have an antitumor potential. The effect of benzimidazole (2 compounds) and azafluorene (13 compounds) derivatives on diaminoxidase and polyaminoxidase activity in a model of cell-free testing system from regenerating liver tissue and hepatoma H-27 was studied. Then the influence of the compounds shown antineoplastic properties at a non-cellular level on proliferative and differentiative activity of murine melanoma B16-F10 cells was evaluated. Carcinostatic properties were manifested by substances 1-amino-4-azafluorenone-9, 1-brom-4-azafluorenone-9 and 1-amino-2-brom-4-azafluorenone-9. These substances can already be considered at this stage of testing as perspective oncoprotective medications.

Chemical Biology as the cornerstone of polyamine research

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Chemical Biology has been recognized as a distinct scientific entity for 10–15 years. This has most probable happened due to recognition of overwhelming complexity of living systems requiring novel rationally developed chemical tools to manipulate distinct metabolic or signaling pathways very precisely. Polyamine metabolism is a rational target for drug design. The key concept for supporting potential drug discovery is increasing knowledge about the specific physiological roles of individual polyamines and a proper understanding of the regulation of polyamine metabolism in mammals and parasites.

Here we present how Chemical Biology is being exploited to study and understand the complexity of polyamine metabolism and precise physiological functions of individual polyamines in living systems. Practical examples including the development of multisubstrate analog inhibitors and novel polyamine analogs are presented. Moreover, a testing platform for systematic studies is being described including some details of current method development. As a summary, Chemical Biology could be regarded as an umbrella covering the synthesis and development of sophisticated chemical tools and systematic and detailed biological studies by experienced scientist in their individual fields.

Novel *C*-methylated spermidine analogs

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The polyamines are ubiquitous multifunctional cations essential for cellular proliferation. Prolonged spermidine depletion diminishes post-translational modification (hypusination) of eukaryotic translation initiation factor 5A (eIF5A) protein, leading to cessation of cell growth.

Here we tested novel β-, γ- and ω-MeSpd as substrates for recombinant spermidine/spermine-N^1-acetyltransferase (SSAT), spermine oxidase (SMO) and acetylpolyamine oxidase (PAO). Both β- and ω-MeSpd were substrates for recombinant SSAT, whereas γ-MeSpd was a competitive inhibitor. γ-MeSpd was not catabolized by PAO, but β- and ω-MeSpd were substrates in the presence of benzaldehyde. None of the analogs was a substrate for SMO.

We also investigated the uptake, metabolism and ability of the analogs to function as hypusine precursor and to support cell growth during difluoromethylornithine (DFMO)-induced prolonged polyamine depletion in DU145 cells. All tested analogs accumulated intracellularly, partly replaced natural polyamines, and supported cell growth for up to 6 days. Among the tested analogs, only γ-MeSpd was not converted into corresponding spermine derivative. β-MeSpd was growth supportive during 12 days of culture with DFMO. 2D-immunoblotting of eIF5A isoforms indicated that β-MeSpd functioned as hypusine precursor.

In conclusion, these novel spermidine analogs represent new tools to manipulate polyamine metabolism, providing new insights into the polyamine targets and regulation mechanisms.

Post-translational *O*-GlcNAc modification of proteins: proteomics to function

Dynamic crosstalk between GlcNAcylation and phosphorylation: roles in signaling and human disease

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O-linked *β*-*N*-acetylglucosamine (O-GlcNAc) serves as a nutrient/stress sensor to modulate metabolic processes and gene expression. O-GlcNAc plays a direct role in the etiology of diabetes, neurodegenerative disease, and cancer. Recent phospho-proteomic and glycomic studies have shown that the crosstalk between GlcNAcylation and phosphorylation is extensive at the individual site level. This dynamic interplay not only occurs by competition at the same or proximal sites, but also by each modification regulating the other's cycling enzymes. For example, several kinases are regulated by GlcNAcylation, and phosphorylation regulates both O-GlcNAc Transferase and O-GlcNAcase. The elucidation of this extensive crosstalk between these two most abundant protein modifications will have a major impact on our view of signaling and transcriptional regulation. Examples of the importance of this dynamic interplay in the regulation of FOXO transcription factors, kinases, RNA polymerase II, and cytokinesis will be presented.

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Protein O-GlcNAcylation: a critical regulator of the cellular response to stress

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Increased levels of O-linked attachment of the monosaccharide *β*-*N*-acetyl-glucosamine (O-GlcNAc) on serine or threonine residues of nuclear and cytoplasmic proteins have typically been implicated as a pathogenic contributor to glucose toxicity and insulin resistance as well as diabetes-related cardiovascular complications. However, there is a growing body of data demonstrating that acute activation of O-GlcNAc levels is an endogenous stress response associated with enhanced cell survival. Reports on the effect of altered O-GlcNAc levels on the heart and cardiovascular system have grown rapidly over the past five years. Our studies have demonstrated the cardioprotective effects of increased O-GlcNAcylation either by activating O-GlcNAc synthesis with the addition of exogenous glucosamine or preventing its degradation via inhibition of O-GlcNAcase. We found a strong correlation between increased O-GlcNAc levels during reperfusion with both improved contractile function and reduced tissue injury. Immunohistochemical analysis of non-ischemic hearts revealed the expected intense nuclear O-GlcNAc staining but also a strong striated pattern, consistent with O-GlcNAc enrichment of the Z-discs or Z-bands. The Z-disc, a structural element in striated muscle that contributes to transmission of force generated by myofilaments, also plays a critical role in mechanotransduction signaling pathways. Co-localization of O-GlcNAc with two Z-disc proteins, desmin and vinculin was used to confirm enrichment of O-GlcNAc at the Z-disc. Subsequent studies have shown that vinculin appears to modified by O-GlcNAc and that O-GlcNAc transferase, which catalyzes O-GlcNAc synthesis is directly associated with both vinculin and desmin. In conclusion, these results support the notion that that acute increases in overall myocardial O-GlcNAc levels affords remarkable ischemic cardioprotection, which is associated with O-GlcNAcylation of key proteins that may contribute to the observed protection.

Metabolism and O-GlcNAc modification: mechanisms and regulation

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O-linked *N*-acetylglucosamine (O-GlcNAc) is a post-translational modification of proteins that has been shown to regulate several metabolic pathways. Although original work in the field emphasized the pathway as a mediator of the deleterious effects of excess glucose, more recent work has demonstrated that the pathway serves an important role in normal glucose concentrations also. For example, overexpression of O-GlcNAcase in the livers of euglycemic mice significantly increased the activity of Akt in the insulin signal transduction cascade, resulting in increased phosphorylation and regulation of its downstream targets such as glucose-6-phosphatase. Furthermore, recent work has also indicated that the pathway regulates systems other than metabolic ones, such as angiogenesis and vascular sprouting. Thus, the pathway has grown from one that was originally discovered as a mediator of "glucose toxicity" to one that needs to be considered in all studies of posttranslational modification of all proteins.

Consistent with its broad role in cellular regulation, the pathway is regulated in a complex fashion. At high levels of glucose flux, much O-GlcNAc modification is substrate limited, but we also discovered a significant induction of O-GlcNAc modification of a limited number of proteins under conditions of glucose deprivation that is mediated by upregulation of mRNA for nucleocytoplasmic O-linked *N*-acetylglucosaminyltransferase (ncOGT). The signal for this induction does not appear to be general energy depletion but rather decreased cellular hexosamine flux. These findings suggest a novel negative feedback regulatory loop for OGT and O-GlcNAc regulation and further underline the importance to general cell function in tight regulation of O-GlcNAc signaling.

Studies of O-GlcNAc processing enzymes aid the generation of probes for studying the role of O-GlcNAc

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2-acetamido-2-deoxy-beta-D-glucopyranose residues O-linked (O-GlcNAc) to serine and threonine residues of nucleocytoplasmic

proteins are a common post-translational modification found in higher eukaryotes. Two enzymes play direct roles in regulating intracellular O-GlcNAc levels; a glycosyl transferase that mediates installation of O-GlcNAc (O-GlcNAc transferase, OGT) and a glycoside hydrolase (O-GlcNAcase, OGA) that acts to remove this modification from proteins. Here we describe our efforts to understand the biochemistry and biological roles of these two enzymes. Aspects of the catalytic mechanisms of these enzymes are discussed and how this knowledge has enabled generation of potent inhibitors of OGA that are highly selective for this enzyme over functionally related glycoside hydrolases. We show these OGA inhibitors are effective both in cultured cells and in vivo and we describe some preliminary studies making use of these compounds.

O-GlcNAc, a new player involved in Alzheimer's disease

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Alzheimer's disease (AD) is a progressive neurodegenerative disease and the major cause of dementia in adults. Neurofibrillary degeneration plays a pivotal role in the pathogenesis of AD and is characterized by abnormal hyperphosphorylation and aggregation of tau protein as neurofibrillary tangles in the brain. Tau is a phosphoprotein and is also modified by β-*N*-acetyl-glucosamine (GlcNAc), which is a unique type of O-glycosylation called O-GlcNAcylation. We found that O-GlcNAcylation regulated phosphorylation of tau inversely both in vitro and in vivo. In AD brain, major brain glucose transporters were decreased, and glucose uptake/metabolism is impaired. Decreased glucose metabolism resulted in decreased O-GlcNAcylation and consequently hyperphosphorylation of tau in the mouse brain. In AD brain, O-GlcNAcylation is decreased and tau phosphorylation is increased. The decrease in O-GlcNAcylation correlated negatively to hyperphosphorylation of tau in human brain. Abnormally hyperphosphorylated tau isolated from AD brain contained much less O-GlcNAc modification than the non-hyperphosphorylated tau. Taken together, we propose that in AD, impaired glucose uptake/metabolism results in decreased O-GlcNAcylation and consequently hyperphosphorylation of tau, leading to neurofibrillary degeneration. Therefore, tau O-GlcNAcylation is a new key player linking between impaired brain glucose metabolism and AD.

O-GlcNAc: friend or foe in feast or famine

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The cellular response to feast or famine is mediated by the concerted action of a variety of key signaling pathways including the AMP-kinase, mTOR and Hexosamine-signaling pathways. These pathways interact with, and serve to modulate, homeostatic mechanisms such as the Insulin signaling, TGF-β and MAP-kinase signaling cascades. The hexosamine-signaling pathway is of particular interest since it is responsive to cellular levels of amino acids, sugars and ATP. The enzymes of O-GlcNAc cycling are recruited to their sites of action by the same activation mechanism (PI-3 kinase) triggering Insulin and many other signaling cascades. Thus, the Hexosamine-signaling pathway impacts Insulin signaling and other pathways by directly responding to nutrient availability. Our genetic evidence further suggests that Hexosamine-signaling by O-GlcNAc serves as an epigenetic modulator of transcription, translation, and protein stability. The two key enzymes in this process, O-GlcNAc transferase and O-GlcNAcase, have emerged as promising drug targets. The pathways impacted by the nutrient-responsive hexosamine-signaling pathway modulate key physiological processes dysregulated in metabolic syndrome (stress, innate immunity, and metabolism). In fact, the O-GlcNAcase gene is a known diabetes susceptibility locus in Mexican Americans. A 'vicious cycle' exists in such populations; children of mothers with diabetes show increased risk for developing the disease due to unknown epigenetic factors in the intrauterine environment. We have modeled defects in the O-GlcNAcase gene by creating an O-GlcNAcase knockout mouse. Our current hypothesis is that O-GlcNAc cycling integrates metabolic information, potentially leading to epigenetic reprogramming in the intrauterine environment.

The *Xenopus laevis O*-GlcNAcome: *O*-GlcNAc transferase is needed for the G2/M transition

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O-linked *N*-acetylglucosaminylation (*O*-GlcNAcylation) is an abundant and essential post-translational modification confined within the nucleus and the cytosol. This glycosylation is highly dynamic and this dynamism is closely controlled by two antagonist enzymes named *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase. *O*-GlcNAcylation is directly linked to glucose metabolism through the hexosamine biosynthetic pathway which regulatory key-enzyme is the glutamine:fructose-6-phosphate amidotransferase (GFAT). Because of its high reversibility, *O*-GlcNAcylation is often compared to phosphorylation of which it could counteract the effect by modifying either the same amino-acids or adjacent amino-acids. Recent studies have demonstrated that *O*-GlcNAcylation take part in cell cycle regulation. Using full-grown *Xenopus* oocytes, that present the particularity to be arrested at the prophase of the first meiotic division, we showed that progesterone-stimulated G2/M transition was characterized by an increase in *O*-GlcNAcylation and that OGT inhibition prevented oocytes to resume meiosis. OGT inhibition prevented germinal vesicle breakdown, and both progesterone stimulation and egg cytoplasm injection-activated MPF and MAPK pathways. Alternatively to OGT inhibition, GFAT inhibitors were used but failed to prevent GVBD. Such strategy appeared to be not relevant because assays of UDP-GlcNAc pools in matured and immature oocytes revealed a constant concentration of the nucleotide-sugar. Finally, we identified 25 *Xenopus O*-GlcNAcylated proteins among which erk2 (MAPK), actin, glycolytic enzymes and ribosomal proteins suggesting that *O*-GlcNAcylation regulates cell homeostasis at many levels. These studies also reinforce the crucial role for *O*-GlcNAcylation in G2/M transition and suggest that its function is requested in cell cycle regulation.

Synaptic O-GlcNAc proteomics and function in normal versus Alzheimer's disease states

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We recently reported proteomic identification of site-specific O-GlcNAc modifications at neuronal synapses, particularly on proteins that regulate distribution of synaptic vesicle pools. We examined potential roles for O-GlcNAc in mouse hippocampal synaptic transmission and plasticity through in vivo pharmacological modulation of O-GlcNAc and analysis of biochemical signaling, electrophysiology, and behavioral learning/memory. Hippocampal O-GlcNAc levels increased rapidly in response to neuronal depolarization and whole animal spatial learning. Pharmacological elevation or reduction of O-GlcNAc levels had no effect on Schaffer collateral CA1 basal hippocampal synaptic transmission. However, in vivo elevation of O-GlcNAc levels enhanced long term potentiation (LTP), an electrophysiological correlate to some forms of learning/memory. Reciprocally, pharmacological reduction of O-GlcNAc levels blocked LTP. Elevation of O-GlcNAc levels led to increased phosphorylation of Synapsin I/II and activation of Erk 1/2, events previously linked to establishment of LTP. Reduced O-GlcNAc has been associated with Alzheimer's disease. In the Alzheimer's mouse model 3XTg-AD, we observed reduced O-GlcNAc on specific synaptic proteins, including synapsin I. Pharmacological elevation of O-GlcNAc in 9 months 3XTg-AD mice normalized defects in cognitive hippocampal dependent spatial learning, and this was accompanied by modulation of several LTP linked phosphoryaltion events. Thus, O-GlcNAc is a novel regulatory signaling component of neuronal synapses, with specific roles in plasticity that involve interplay with phosphorylation, and may represent a novel therapeutic target for improvement of synaptic deficits and memory in Alzheimer's disease.

Proteasomal proteomics

Proteasomes: cellular nanomachines for processing and breakdown of proteins

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Proteasomes are the major enzymes responsible for catalysing the degradation of intracellular proteins. The 20S proteasome is a 10×15 nm cylinder-shaped particle built up by 4 stacked seven membered rings consisting of alpha- and beta-subunits with a stoichiometry $\alpha 7 \beta 7 \beta 7 \alpha 7$. It contains a central inner chamber, where three β-subunits ($\beta 1$, $\beta 2$, $\beta 5$) contain the active sites for peptide bond hydrolysis. Associated to one or both α-rings of the 20S proteasome are multisubunit 19S regulatory particles that are able to bind proteins marked as proteasome substrates by prior conjugation with polyubiquitin chains, and initiate unfolding, deubiquitination and translocation of the substrates into the proteolytic chamber of the 20S proteasome. Under conditions of intensified immune response, eukaryotic cells adapt their proteasomes by replacing the standard catalytic β-subunits by immuno catalytic β-subunits (i$\beta 1$, i$\beta 2$, i$\beta 5$). Additionally a proteasome activator complex, PA28, can be generated and associated to one or both α-rings. Both of these adaptations change the protein-breakdown process for optimized generation of antigenic epitopes that are presented by MHC class I complexes. Partial replacement of standard β-subunits by immuno β-subunits leads to formation of intermediate-type 20S proteasomes. Hybrid proteasomes (19S regulator—20S proteasome—PA28) may have special functions within the numerous functions proteasomes have been found to be responsible for in eukaryotic cells, e.g. regulation of cell proliferation and cell differentiation, immune- and stress-response, metabolic regulation ad many others.

Proteomic analysis of co- and post-translational modifications in the yeast 26S proteasome

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Identification and functional analysis of co- and post-translational modifications is crucial for a better understanding of functions of proteins and protein complexes. We purified the yeast 26S proteasome, consisting of two 19S regulatory particle (19S RP) (2×18 subunits) and 20S proteasome (28 subunits) and detected almost comprehensively its co- and post-translational modifications by mainly mass spectrometry such as MALDI-TOF/TOF MS and ESI-Q/TOF MS. A total of 84 subunits with co- or post-translational modifications, including *N*-acetylation, *N*-myristoylation, phosphoryation and a single *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) modification, were identified in the 26S proteasome. A total of 22 and 18 *N*-acetylated subunits were detected in 19S RP and the 20S proteasome, respectively. By using *N*-acetyltransferase deletion mutants, we found that the chymotryptic activity and accumulation level of the 20S proteasome from the *N*-acetyltransferase deletion mutant were significantly higher than in those from the normal strain. On the other hand, we detected 18 phosphorylated subunits of the 26S proteasome and identified a total of 40 Ser/Thr phosphorylation sites using mainly immunobilized metal affinity chromatography, followed by MS/MS analysis. Dephosphorylation treatment of the 19S RP with phosphatase resulted in a decrease in ATPase activity of 19S RP and chymotryptic activity of the 20S proteasome. Furthermore, we identified modifications with a single O-GlcNAc on 10 and 12 subunits in 19S RP and the 20S proteasome, respectively using deglycosylation and Western blot analysis. Several O-GlcNAc-modified subunits were phosphorylated, suggesting that O-GlcNAc might regulate phosphorylation by competition for binding to phosphorylation sites.

Efficient workflows for the validation and quantitation of protein and peptide biomarker candidates

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A large number of laboratories around the world are identifying protein and peptide biomarker candidates with proteomics approaches. However, very few of these candidates have been validated. The main reason is the unsuccessful transition from the discovery phase

(non-targeted proteomics) to the validation phase (targeted proteomics). Candidates in the validation phase need to be quantitated reliably in a large number of samples with sufficient speed, reproducibility and sensitivity. Most approaches cannot achieve this in matrices as complex as serum or plasma.

The use of mTRAQ™ reagents in combination with a global standard or quantitation with isotopically coded proteotypic peptides utilizing MRM's offers the speed, sensitivity and specificity necessary for validation of the biomarker candidates in complex matrices. Several examples of published and ongoing studies will be presented.

Protein species: the big challenge for the next centuries

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The term protein species describes protein variants, which are coded by one single gene, but vary in their exact chemical composition. The term protein species covers splicing variants, truncated proteins and posttranslational modified proteins. The importance of the knowledge of the exact chemical composition of a protein species is given by the relationship between its function and its composition. Since several centuries it is known that phosphorylation is a switch turning on or off the activity of enzymes. Truncations can activate receptors, proteases or peptide hormones. In contrast to this knowledge the relationship of the exact chemical composition of a protein and its function is not yet fully taken into account in many investigations of proteins. In many of the past proteomics approaches protein identification relies on sequence coverage significantly below 100% and posttranslational modifications are paid no attention to. A second obstacle concerning the description of protein species derives from the absence of a nomenclature, which fully describes its exact chemical composition. An internationally accepted and applied unique nomenclature for proteins is still missing. Most important in the context of protein species is that none of these synonyms have a relationship towards defined protein species. In summary the total analysis of the chemical composition of a protein species is already a big challenge, however it is even more challenging to develop strategies for the validation of the correctness of the function—exact chemical composition relationship. Furthermore a unique nomenclature for the description of protein species is urgently needed.

Catalytic subunit LMP2 of stress-induced immunoproteasomes is crucial in the pathogenesis of enterovirus myocarditis

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Murine models of coxsackievirus B3 (CVB3)-induced myocarditis mimic the divergent human disease course of cardiotropic viral infection. Immunoproteasomes (IP) are crucial in the modulation of adaptive immune responses, in the maintenance of protein homeostasis and in the preservation of cell viability under stress conditions. Our previous work has established that IP expression in the infected myocardium is linked to a strong enhancement of viral epitope generation.

We investigated the impact of IP function in enterovirus myocarditis. Mice, which are deficient in immunosubunit LMP2 of the stress-induced IP, were infected with $1 \times 10e^5$ PFU CVB3 Nancy strain. In concurrence to wt littermates, we observed a pronounced up-regulation of cardiac IP subunit LMP7 as early as day 4 p.i. in LMP2-deficient mice. However, LMP2-deficiency was linked to less severe myocarditis at day 8 p.i. (HE stain of cardiac tissue sections: wt 1.95 ± 0.20 versus LMP2-deficiency 0.71 ± 0.06 (grade of myocarditis; scale 0–4; $P < 0.001$). Whereas the cardiac output (CO) was reduced in wt littermates in enterovirus-myocarditis ($P < 0.05$), there was no difference in LMP2-deficient mice in comparison to sham-treated mice. Maximal left ventricular pressure and $dPdt_{max}$ were impaired in acute myocarditis in wt littermates. In contrast, systolic function was not affected by CVB3 infection in LMP2-deficient mice. Likewise, diastolic function was preserved in LMP2-deficient mice upon enterovirus infection. Our findings of less severe myocarditis in LMP2-deficient mice were associated with reduced viral load in the myocardium of this strain. In wildtype mice, myocardial LMP2-expression is up-regulated at stages of acute myocarditis. Immunoblot analysis revealed enhanced LMP7-incorporation in both wildtype littermates and LMP2-deficient mice: LMP7 was detected at similar levels in both strains.

In conclusion, this study suggests an impact of LMP2-immunosubunit function in regulatory processes of viral replication. LMP2-deficiency is associated with less severe myocarditis upon CVB3 infection.

Protective role of calcium and glutamate receptors

Effects of Ca^{2+}-permeable AMPA receptors by genetically impaired RNA editing in the mouse

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Genetic studies illustrate that elevated Ca^{2+} in the central nervous system does not always lead to neurotoxicity. In fact, Ca^{2+} can protect neurons from dying depending on the age of the animal, route of elevation, timing of insults, and brain regions involved. For example, genetic manipulation of the Q/R site-edited gene of the GluR-B (GluA2) subunit of AMPA receptors in the embryo leads to spontaneous seizures and premature death. However, in the adult brain, no neuropathological symptoms develop when GluA2(Q) expression is restricted to postnatal hippocampal neurons. Moreover, the ablation of GluA2 alleles results in the Ca^{2+} permeability of all remaining AMPA receptors in the CNS. At the same time, the lack of GluA2 negatively affects the synaptic AMPA receptor levels, and thus, excitatory transmission strength decreases and the Ca^{2+} influx via the remaining AMPA receptors does not generate cell death. Mice lacking GluA2 in forebrain are impaired in spatial learning in absence of signs of neurotoxicity. Similarly, a mutation of the NR1/NR2A subtype of NMDA receptors causing Ca^{2+} influx at the neurons' resting potential due to failure of the voltage-dependent Mg^{2+} block leads to memory deficits but no cell death in hippocampus or forebrain structures. However, spinal cord motoneurons may be particularly sensitive to sustained Ca^{2+} influx by AMPA receptors, especially as a consequence of suboptimal Q/R site editing.

Protective effects of early-life seizures in vivo and early exposure of excitatory amino acids in vitro

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Although abnormal rises in intracellular calcium $[Ca^{2+}]_i$ can kill neurons Ca^{2+} can also prevent neurons from dying. For example, postnatal (P) day P20 rats are sensitive to CA1 injury following a single injection of kainic acid (KA) (1xKA) but resistant to injury when animals have a history of seizures on P6 and P9 (3xKA). In vivo and in vitro approaches were taken to get a better understanding of this type of neuroprotection. In vivo experiments showed partial sparing of dendritic spines and branching on CA1 pyramidal neurons after 3xKA compared with 1xKA using Golgi Technique. Microarrays of the CA1 subregion and immunohistochemistry in culture showed distinct calcium-binding protein families (annexin 3) and anti-apoptotic Bcl-2 gene members were increased after 3xKA or two exposures to NMDA (parvalbumin). Ca^{2+} imaging studies showed that NMDA responses were enhanced at 5 h after 1xKA but attenuated after 3xKA. Decreases in Ca^{2+} permeability coincided with early reductions in NR1 subunit protein expression in CA1 neurons compared to loss of GluR2 subunits previously reported in adults. In vitro studies where we developed a "two hit" model in hippocampal cultures showed pretreatment with high doses of glutamate or NMDA at young ages (5 DIV) followed by a brief exposure to a second high dose 9 days later also spared a significant population of neurons compared to one exposure at 14 DIV. In vivo and in vitro data suggest that early-life exposure to glutamate or other excitatory amino acids, even at high doses, may protect against subsequent insults.

Neuroprotective effects of moderate increases in intracellular calcium

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Large increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$)in neurons during hypoxic/ischemic stress causes cell death. However, new evidence shows that smaller increases in $[Ca^{2+}]_i$ initiate survival responses. Evidence includes the neuroprotective effects of small concentrations of Ca^{2+} ionophores (increasing $[Ca^{2+}]_i$ by ca. 50 nM), demonstration that anoxia tolerant neurons (neonatal mammals, turtles, frogs, hibernators) require moderate increases in $[Ca^{2+}]_i$ for surviving prolonged anoxia, and demonstration that the neuroprotective effects of anesthetics require increases in $[Ca^{2+}]_i$. Further, in organotypic cultures of rat hippocampus, hypoxic preconditioning requires IP_3 receptor-dependent Ca^{2+} release from the ER via increased cytosolic NAD(P)H. Ca^{2+} chelation with intracellular BAPTA, ER Ca^{2+} store depletion with thapsigargin, IP_3 receptor block with xestospongin, and IP_3 receptor RNA interference all blunt the moderate increases in $[Ca^{2+}]_i$ required for preconditioning. Increases in $[Ca^{2+}]_i$ during preconditioning and neuroprotection are not prevented by NMDA antagonists or by removing Ca^{2+} from the bathing medium. Demonstration that NADH manipulation increases $[Ca^{2+}]_i$ in an IP_3R-dependent manner reveals a primary role of cellular redox state in liberation of Ca^{2+} from the ER. Blockade of IP_3Rs and intracellular Ca^{2+} chelation prevent phosphorylation of known HPC signaling targets, including MAPK p42/44 (ERK), protein kinase B (Akt) and CREB. Therefore, the endoplasmic reticulum, acting via redox/NADH-dependent intracellular Ca^{2+} store release, is an important mediator of the neuroprotective response to hypoxic stress. Small increases in $[Ca^{2+}]_i$ may be a general signal that produces adaptive/pro-survival responses to stress in neurons and probably other cells.

Neurodegeneration induced by blockade of activity: role of glutamate receptors

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Spontaneous neuronal network activity is an essential attribute of the developing nervous system. Such activity is also found in primary neuronal cultures, which we use to investigate the possible effects of manipulation of network activity on neuronal survival. Chronically silenced (using TTX) cultured cortical neurons undergo a process of progressive cell death over a period of up to two weeks. Paradoxically, blockade of glutamate receptors in the TTX silenced neurons protected them from death. In a search for the mechanisms underlying this slow cell death, we found that the neurons initially reduce expression of GluR2, and the altered balance between GluR1 and GluR2 is likely to result in a rise of intracellular calcium concentration in response to spontaneous synaptic activity that is still present in the network-silenced neurons. Indeed, we found that the TTX treated cells have a higher ambient intracellular calcium concentration than controls. We further explored the mechanisms underlying this neuronal death and found that calpain, a calcium dependent protease, is activated in the TTX treated neurons. Indeed, an endogenous blocker of calpain, calpastatin, significantly attenuated TTX-induced dendritic degeneration and eventual neuronal death. We propose that neurons are unable to cope with otherwise nontoxic influxes of calcium ions associated with spontaneous synaptic currents when they are devoid of GluR2 receptors. These results concur with the hypothesis regarding the involvement of GluR2 receptors in neuroprotection and its absence in triggering the apoptotic process induced by blockade of spontaneous activity.

Protein interactions in the virus–host relationship Molecular mechanisms of flavivirus membrane fusion

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Flaviviruses are small enveloped viruses that enter cells by receptor-mediated endocytosis and release their nucleocapsid into the cytoplasm by fusing their membrane with the endosomal membrane. The fusion event is triggered by the acidic pH in the endosome and is mediated by the major envelope protein E. Based on the atomic structures of the pre- and post-fusion conformations of E, a fusion model has been proposed that includes several steps leading from the

metastable assembly of E at the virion surface to membrane merger and fusion pore formation. Using tick-borne encephalitis virus as a model, we have defined individual steps of the molecular processes underlying the flavivirus fusion mechanisms. This includes the identification of a conserved histidine as the pH sensor in the fusion protein that responds to the acidic pH in endosomes and thus initiates the structural transitions driving fusion.

Peptides derived from HIV-1 protease inhibit Vif activity

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Vif (virion infectivity factor) is essential for the human immunodeficiency virus type 1 (HIV-1) propagation in the natural target cells. These cells express a cellular cytidine deaminase APOBEC3G (A3G), which inhibits the production of infectious HIV-1. Vif mediates A3G degradation by the proteasome. Thus, Vif is an attractive target for developing anti-HIV-1 drugs.

We have reported that PR1-9 peptide derived from the N-terminus of HIV-1 protease inhibits Vif function in restrictive cells. In this work we found that PR2-5 is a minimal peptide, which inhibits the production of infectious HIV-1 in restrictive cells. The amino acid residues at positions 2(Q), 4(I) and 5(L) are essential for PR1-9 inhibitory effect, and substitutions of 2(Q) for (N) and 5(L) for (V) improve the efficacy of the peptides as HIV-1 inhibitors. Moreover, the introduction of positively charged residues, either at the N-terminus [3 K or $(Me)_2$N-R-] or at the C-terminus (3 K) of the peptide, showed a significant improvement in the peptide's activity. A very high level of inhibition was also observed when two segments of PR1-5 were linked through a spacer.

PR2-5 serves as a leading compound for developing a new class of HIV-1 inhibitors.

Caught in the act: optical tools for the visualization of proteins and replicating viral genomes in single living cells in real time

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Optical tools that allow dynamic measures in living cells are increasingly being exploited to gain information on specific viral processes. These methods range from fluorescence recovery after photobleaching, to study protein mobility and diffusion kinetics, to fluorescence resonance energy transfer (FRET) to study protein interactions.

Viral RNA biogenesis is a crucial step in the replication of RNA viruses and retroviruses that require both the production of genomic RNAs and of translation templates. Cellular and viral factors concur in the biogenesis of RNA at the specific sub-cellular site where the reaction takes place. The possibility of tracking viral RNA in living cells gives the unique possibility of measuring the kinetic parameters of RNA biogenesis as well as defining the dynamic recruitment of host and viral factors to the site of replication.

By engineering a RNA-tagged human immunodeficiency (HIV-1) retrovirus we could characterize the HIV-1 transcription cycle allowing precise kinetic measurements of RNA polymerase elongation rates as well as initiation, splicing and termination steps (Boireau, et al. JCB 2007). In addition we also analyzed the dynamic of the TAR:Tat:pTEFb complex at the site of HIV-1 transcription in living cells (Molle et al. in Retrovirology, 2007). Our data suggest that this complex dissociates from the polymerase following transcription initiation, and may undergo subsequent cycles of association/dissociation.

We extended this approach to the tick-borne encephalitis virus (TBEV). Flaviviruses are positive RNA viruses that assemble the replication complex in the cytoplasm of the infected cells (Miorin et al. 2008). The modified TBEV replicons were competent for RNA replication and allowed the visualization of replicated genomic RNA that accumulated in cytoplasmic structures with a distinct sub-cellular localization. This work provides for the first time a kinetic framework to analyze viral RNA biogenesis and RNA/protein dynamics in living cells.

This advance, coupled to the visualization of protein interactions by FRET would allow careful dissection of viral replication complexes in single cell and in real time.

Design and synthesis of new peptidomimetic artificial ribonucleases with antiviral activity

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The synthesis of molecules that are capable of nonrandom RNA cleavage has found a variety of important applications in molecular biology. For instance, such molecules are used as structural probes for nucleic acids in solution, or in rational design of novel anti-infective agents, since RNA is the genetic material of many pathogenic viruses.

Here we represent design and synthesis of peptide-like molecules mimicking the catalytic site of natural ribonucleases (A and T1):

Series 1: These dipeptides contain O-methyl or decyl ethers of hystidine and one of following amino acids: Lys, Tyr, Thr, Ser;
Series 2: Tetrapeptides Glu–X–Arg–Gly–OAlkyl;
Series 3: Tetrapeptides Glu–X–Lys–Gly–OAlkyl; X–Gly, Ala, 4-aminobutyric acid, 6-aminohexanoic acid, *p*-aminobenzoic acid;
Series 4: These compounds are symmetric peptide-like molecules in which amino acids (Lys, Glu, Ser, His) are connected by linker group of the various nature.

Ability of artificial RNases to RNA cleavage was shown in experiments with 96-mer RNA HIV-1. Anti-influenza activity and cytotoxicity of 15 peptidomimetics from series 1–4 was investigated. Some of the tested compounds exhibited significant antiviral effect and showed low cytotoxicity.

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Protein phosphorylation and quantitation by mass spectrometry

Integrative network analysis of cell-specific Eph/ephrin phospho-tyrosine signaling

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In multicellular organisms, cell sorting is crucial in order to obtain and maintain tissue-structure and -boundaries. Signaling through the Eph receptor—ephrin global positioning system is essential for these processes. Both the Eph receptor and the ephrin initiate a tyrosine kinase dependent signal, suggesting that coordinated bidirectional signaling between interacting Eph and ephrin expressing cells is important to regulate cell-sorting.

We have developed a novel proteomic approach, *q*uantitative analysis of *bid*irectional **s**ignaling (qBidS) to investigate signaling between mixed cell lineages. Stable isotopomeric versions of Argine and Lysine were used to label cell lineages expressing Eph receptor or ephrin respectively. Mixing of these lineages initiates contact dependent signaling in both cells, which subsequently was analyzed in a lineage specific and quantitative manner. We identified 101 tyrosine phosphorylation events common to both Eph receptor and ephrin expressing cells. Interestingly, 72% of the tyrosine phosphorylations were modulated in a cell specific manner. In addition, we carried out a siRNA screen for signaling molecules involved in EphR-ephrin controlled cell sorting and identified 200 targets. Finally, we used integrative and data-driven network modeling which revealed cell specific network structure and kinase utilization. In summary, we have identified novel and cell specific regulatory mechanisms essential for cell sorting.

Use of label-free relative quantitative mass spectrometry to determine kinase and ubiquitylation post-translational modification substrate specificity

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In the last couple of years there has been a significant increase in the use of mass spectrometric based quantitation to measure global changes in proteomics experiments. Most researchers have relied upon isotope labelling methods such as stable isotope labelling of amino acids in cell culture (SILAC) and Isobaric tagging for relative and absolute quantitation (ITRAQ) as multiple test samples can be combined prior to mass spectrometric analysis. Although powerful, these methods have a limited dynamic range for quantitation on the peptide level, requires substantial sample processing prior to mass spectrometric analysis and are relatively expensive. The development of mass spectrometers with high resolution and mass accuracy coupled with HPLC that have reproducible retention times has allowed the use of this technology also to be applied to label-free quantitation based on monitoring changes in ion abundances. This technique is gaining in popularity as sample preparation is simple, inexpensive and has a higher dynamic range than isotope labelling methods. This method is also advantageous in that it is possible to perform preliminary quantitation analyses on mass spectrometric data that have been previously acquired without quantitation in mind. We have applied label-free quantitative mass spectrometry using our Agilent 6510 QTOF to analyse dynamic changes in post-translational modification from both in vitro and in vivo experiments on the protein and peptide level. Here we show examples of how label-free quantitation can be used to determine kinase and ubiquitylation specificity on *PAK4* (p21 protein (Cdc42/Rac)-activated kinase 4) and Lyric (metadherin), respectively.

Characterisation and quantitation of neuronal membrane rafts after kinase inhibition and toxic insults in Alzheimer's disease

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Membrane rafts are cholesterol and sphingolipid-rich membrane microdomains that have critical roles in diverse cellular functions. Recently, the direct involvement of neuronal membrane rafts has been implicated in several neurodegenerative disorders. As part of our efforts to investigate the involvement of membrane rafts in Alzheimer's disease (AD), the preparation of membrane rafts from neuronal cells using a panel of detergents was evaluated. Preparations using the detergent CHAPSO were found to isolate cholesterol-rich low density rafts most effectively. Moreover, proteomic profiling and semi-quantitative analysis of the CHAPSO preparation revealed a far richer protein population with a higher recovery of membrane and lipid-anchored proteins compared to the traditionally used Triton-X100 preparation. Distinct categories of proteins were also enriched in the CHAPSO preparation including channels and receptors, synaptic proteins, and rab signalling proteins. Importantly for neuronal analyses, AMPA and GABA receptors known to cluster in rafts and several proteins implicated in neurological disorders were also clearly enriched by CHAPSO but not by Triton-X100.

In relation to AD, neuronal membrane raft proteins sensitive to cellular treatment with the pathogenic AD peptide Aβ, or the inhibition of the kinase GSK3 implicated in both tau and Aβ pathology, were quantified by a hybrid proteomic approach using iTRAQ tags. This method was six times more sensitive than a traditional in-solution digestion approach more typically used for iTRAQ experiments, and enabled the quantitation of three times more proteins. Quantitation of the raft proteins revealed some clear changes after Aβ treatment but more subtle alterations upon GSK3 kinase inhibition.

Analysis of in vivo histone methylation kinetics by Heavy Methyl SILAC

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Histone methylation has been long considered an epigenetic mark which mediates the inheritance of chromatin functional states, basing on the assumption of its irreversibility. The discovery of de-methylase activities revolutionized this generally accepted concept proposing the reversibility of methylation at histones. As such, an approach for the determination of in vivo histone methylation is of tremendous biological importance, though extremely challenging.

We describe a novel proteomics approach which accomplishes this daunting task by a combination of heavy methyl SILAC (hmSILAC) labeling and unbiased identification of modifications based MaxQuant software. HmSILAC is variation of SILAC, where methylation is labeled instead of proteins, which already proved big potential to detect and quantify methylations in vivo. We performed time course experiments in HeLaS3 cell lines and in murine embryonic fibroblast (MEFs) derived from mice double null for different methyltransferases, such as Su(var)4-20and/or Su(var)3-9.

Methylation kinetics where simultaneously determined for several methylation sites involved in both activation (H3K4, H3K36, H3K79), as well as repression (H3K9, H3K27, H4K20) of transcription: mono-methylation had the fastest heavy methylation incorporation, with no significant differences among sites. Instead, higher-methylation states (di-, tri-) showed a distinction between sites/marks functionally linked to either gene activation or gene repression, with "active" sites having significant faster turnover. Studies on Su(var)4-20 knock-out showed the expected change in methylation pattern (reduction in H4K20me2 and H4K20me3, increase in H4K20me), together with a reduction in the kinetics of the reaction 'H4K20 to H4K20me'. Parallel results for Su(var)3-9 mutant will be described.

Thus, the novel combined proteomics approach presented can provide a tool to measure histone methylation kinetics in vivo, with a major impact upon epigenetic research.

Regulation of AMPA/kainate receptors

AMPA receptor aptamers

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The α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) subtype of glutamate ion channel receptors plays an important role in the mammalian brain functions such as memory and learning, whereas excessive receptor activity has been implicated in some neurological disorders and diseases such as stroke, epilepsy, and amyotrophic lateral sclerosis. Inhibitors against AMPA glutamate receptors are therefore drug candidates for potential treatment of these neurological diseases. Using systematic evolution of ligands by exponential enrichment (SELEX), we have successfully identified three different classes of RNA inhibitors or RNA aptamers with nanomolar affinity against the GluR2Qflip receptor, a key AMPA receptor subunit that controls the calcium permeability and mediates excitotoxicity. One class is a group of competitive aptamers, which we selected by using NBQX, a classic competitive inhibitor. The highest potency or IC50 value for one of the aptamers in this class reached 30 nM, rivaling any exiting AMPA receptor inhibitors. We have also identified two other classes of aptamers that are selective to different conformations of GluR2Qflip: one class uniquely inhibits the open-channel conformation whereas the other inhibits the closed-channel conformation. Therefore, our results suggest that developing water-soluble, nanomolar affinity aptamers that are selective to an AMPA receptor subunit and even to a unique conformation of that subunit is possible. Furthermore, these aptamers are alternative templates for design of better inhibitors and better drug candidates against AMPA receptors.

Regulation of cellular biosynthetic events

Akt and S6 Kinases in growth and nutrient homeostasis

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Mammalian target of rapamycin (mTOR) plays an evolutionary conserved role in the control of organismal growth depending on nutrient availability. In the last few years our laboratory has investigated the functions of the mTOR substrates, Akt and S6 kinases. I will present evidence that Akt2 and S6K1 have complementary roles in the control of insulin action and insulin production. The combined deletion of both genes in mice recapitulates features of type 2 diabetes and alters the response to high fat diet. Next I will address the contribution of Akt and S6 kinases on cell cycle progression and tumorigenesis in two models of liver growth: (1) liver regeneration after partial hepatectomy; (2) hepatic adeno-carcinoma formation due to deletion of the tumour suppressor PTEN. Our results indicate striking functional specificity of these kinases in tissue growth and metabolism.

A novel mechanism by which amino acids regulate the protein synthesis machinery

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Eukaryotic initiation factor 2B (eIF2B) is a heteropentameric guanine nucleotide exchange factor whose function is to regenerate active eIF2.GTP between successive cycles of translation initiation. eIF2 recruits the initiator methionyl-tRNA to the ribosome, and is therefore needed for the initiation of translation of every cytoplasmic mRNA.

eIF2B thus plays a key role in controlling the initiation of mRNA translation. Depriving human cells of amino acids rapidly results in the inhibition of eIF2B activity, independently of changes in the phosphorylation of its substrate. Although starving cells of amino acids also inhibits signaling through the mammalian target of rapamycin complex 1 (mTORC1), we show that control of eIF2B activity by amino acid starvation is independent of mTORC1.

Instead, amino acids repress the phosphorylation of a previously unknown site in eIF2Bepsilon. We identify this site as Ser525, which is situated next to the known phosphoregulatory region in eIF2Bε. Mutating Ser525 to Ala abolishes the effects of amino acid starvation on eIF2B activity and protein synthesis. Thus, the phosphorylation of this site is crucial for the regulation of eIF2B by amino acids.

These findings reveal a novel pathway in which amino acids regulate translation initiation, which is distinct from other known amino acid-sensitive signaling mechanisms.

How do amino acids regulate mTORC1 signaling?

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Signaling through mammalian target of rapamycin complex 1 (mTORC1) is stimulated by amino acids and insulin. However, it is not clear how amino acids regulate mTORC1. It has been proposed that FKBP38 (immunophilin FK506-binding protein, 38 kDa) inhibits mTORC1 function and that this relieved by its binding to Rheb.GTP, a key activator of mTORC1 signaling. However, we were unable to observe any regulation of FKBP38/mTOR binding by amino acids or insulin. Furthermore, FKBP38 did not inhibit mTORC1 signaling.

The *Drosophila* translationally-controlled tumor protein (TCTP) was reported to act as the guanine nucleotide-exchange factor for Rheb. Depleting TCTP levels did not reproducibly affect mTORC1 signaling in amino acid-replete/insulin-stimulated cells. Overexpressing TCTP did not rescue mTORC1 signaling in amino acid-starved cells. These data also indicate that, in the cell-types tested here, neither TCTP nor FKBP38 regulates mTORC1 signaling.

Accumulation of uncharged tRNA has been proposed to lead to inhibition of mTORC1 signaling during amino acid starvation. To test this, we used a Chinese hamster ovary cell line containing a temperature-sensitive mutation in leucyl-tRNA synthetase. Leucine deprivation markedly inhibited mTORC1 signaling in these cells, but shifting the cells to the nonpermissive temperature did not. These data indicate that uncharged tRNA(Leu) does not impair mTORC1 signaling.

Regulation of cellular functions via the mTOR/S6K pathway

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The mTOR (mammalian target of rapamycin) is central regulator of an evolutionary conserved signalling pathway which controls cellular metabolism, growth and proliferation. Deregulation of mTOR-coordinated signalling has been associated with various human pathologies, including diabetes, inflammation and cancer. Rapamycin, a naturally occurring mTOR inhibitor, and its homologues have been currently tested as anti-cancer drugs in numerous clinical trials. In contrast to yeast which have two TOR genes (TOR1 and TOR2), there is only one gene encoding mammalian TOR. The diversity of TOR-mediated signalling in mammals is compensated by the existence of two multienzyme complexes, mTORC1 and mTORC2, whose regulatory components and downstream effects mirror in part signalling mediated in yeast by TOR1 and TOR2 pathways. The mechanisms by which mTOR sensors and controls energy metabolism and cell growth are relatively well understood, while molecular events defining mTOR-mediated proliferation remain to be elucidated.

We have recently identified a novel mTOR splicing isoform, TORβ, which in contrast to the full length protein (mTORα), has the potential to regulate the G1 phase of the cell cycle and to stimulate cell proliferation. mTORβ is an active protein kinase which mediates downstream signalling through complexing with Rictor and Raptor proteins. Furthermore, overexpression of mTORβ transforms immortal cells and is tumorigenic in nude mice and therefore is a proto-oncogene. These findings define molecular events responsible for mediating mTOR-driven proliferation and oncogenic transformation.

Signalling by amino acid nutrients: mTOR and beyond

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The mTORC1 pathway is regulated by growth factors, hormones and nutrients such as glucose and amino acids. I will discuss: (1) how amino acids regulate the activity of an mTORC1 activator, MAP4K3, and (2) how amino acids signal to another signalling pathway-MEK-ERK.

Amino acids promote regulatory T-loop phosphorylation MAP4K3, at a site required for kinase activity and activation of mTORC1. This site is acutely regulated by amino acids via a PP2A phosphatase.

Innate immune responses to microorgansims are also highly influenced by host nutrient status, although the mechanisms involved are unknown. I will present data indicating that nutrient amino acids signal not only to mTORC1, but also the MAP kinase pathway in macrophages responding to the toll-like receptor 4 (TLR4) ligand, lipopolysaccharide (LPS). We find that a MEK-family kinase regulates both pathways and is itself regulated by amino acid availability. These results suggest an explanation for the influence of host nutrient status on innate immune cell activation.

Effects of leucine-rich diet and treatment with metformin on protein metabolism in tumour-bearing rats

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Background: Cancer induces cachexia state due to nutritional competition between host-tissue and neoplastic cells, characterized by involuntary body mass waste, mainly muscle protein. Leucine improves the protein synthesis in skeletal muscle and modulates the catabolism process in muscles, specially verified in experimental

tumour-being animals. Recent studies have shown that metformin acts as anti-proliferating and anti-angiogenic on cancer treatment.
Methods: Wistar rats were distributed into six groups: control (C), tumour-bearing (W), submitted to leucine-rich diet (3% leucine supplementation) (L), tumour-bearing fed leucine-rich diet (LW), leucine and metformin treatment (100 mg/L in drinking water) (LM), tumour-bearing fed leucine diet, and treated with metformin (LWM). After 15 days, intestinal perfusion analyzed the leucine, methionine and glucose absorption. Total protein content and myosin heavy chain (MHC) expression were analyzed in muscles samples.
Results: MHC expression was lower in W group, although LW has shown similar results as in C group. Moreover, the metformin treated groups showed higher MHC expression compared to control rats. Intestinal absorption of methionine was decreased in W when compared to C, while LWM presented higher absorption than LM and LW. Glucose absorption decreased in all tumour-bearing groups except the LM rats, which presented increase on glucose absorption.
Conclusions: Leucine supplemented diet induced less protein depletion in tumour-bearing animals, and increased, consequently, the nutrients absorption such as amino acids and glucose. Additionally, the metformin treatment promoted higher glucose absorption as well increased the proteins synthesis.

Physical exercise and leucine-rich diet improve the muscle protein metabolism in tumour bearing rats

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Cancer-cachexia induces host waste by increasing the proteolysis and decreasing protein synthesis. Leucine-rich diet can recover the lean body mass preserving the whole muscle protein. Physical exercise can be an excellent alternative of coadjuvant treatment anti-cancer. Knowing this, we examined the effect of leucine-rich diet and exercise on muscle protein metabolism in Walker 256 tumor-bearing rats. Wistar rats submitted to aerobic exercise and leucine-rich diet were distributed into 8 groups: C, control rats; TC, trained, W, tumor-bearing; TW, trained tumor-bearing, L, rats fed leucine diet, TL, trained rats fed leucine diet; LW, tumor-bearing fed leucine diet; TLW, trained tumor-bearing fed leucine diet. After 21 days, the tumor development induced reduction on skeletal muscle protein synthesis in W [3,611 ± 397 nmol/(μg protein h)] and LW [3,014 ± 355 nmol/(μgprotein h)] groups in comparison to C rats [6,870 ± 978 nmol/(μgprotein h)]. Gastrocnemius muscle proteolysis increased in W [2,524 ± 340 nmol/(μgprotein h] compared to C rats [1,189 ± 112 nmol/(μgprotein h)]. The muscle chymotrypsin-like activity increased in W (83.4 ± 24.4 μgprotein/min) compared to C group (49.2 ± 3.8 μgprotein/min); the cathepsin H activity reduced only in TW group (12.7 ± 3.2 μgprotein/min) compared to C (83.3 ± 14.2 μgprotein/min) and the calpain activity decreased in leucine treated groups. The 11S proteasome subunit increased in W (0.137 ± 0.01 arbitrary density) and WT groups (0.141 ± 0.01) compared to C rats (0.027 ± 0.009). Only the W and WL groups showed reduction in the whole myosin content. Although the tumor growth induced protein wasting in skeletal muscle, the effects produced by the association between exercise and leucine-rich diet showed the protein synthesis enhancing and reduced proteolysis, probably improving the tumor-bearing host.
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Repetitive and chronic neurotoxicity: models for understanding long-term deficits affecting the CNS

Basic helix loop helix B2: a new candidate for targeting the brain against ischemia?

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Brain ischemia results in brain dysfunction and overt clinical neurological signs. Glutamate, the neurotransmitter required for normal physiological excitation, is also involved in the pathophysiology of ischemia. Although this neuropathological process can be mediated by any of the glutamate receptors, the *N*-methyl-D-aspartate (NMDA) glutamate receptor subtype plays a major role. In addition, NMDA receptors mediate adaptive responses important for synaptic plasticity during development and in the adult. However, the molecular mechanisms by which NMDA receptors mediate these opposing effects are poorly understood. Brain-derived neurotrophic factor (BDNF) is a member of the family of neurotrophins. BDNF is abundantly expressed and widely distributed in brain, particularly in the hippocampus. BDNF plays a crucial role in neuronal survival, maintenance and synaptic plasticity by binding to its high affinity receptor, TrkB. The *bdnf* gene is complex. There are at least nine exons and a terminal exon, which encodes the mature protein. Promoter IV of the *bdnf* gene is the major promoter that mediates activity-dependent BDNF transcription. Since activity-dependent BDNF expression plays a critical role in neuronal function and dysfunction, we sought to find other transcriptional regulators of promoter IV activity. Here we show that basic helix loop helix B2 (BHLHB2), a member of the basic helix-loop-helix family of transcription factors, represses promoter IV-dependent BDNF gene transcription. BHLHB2 is expressed during development when there is ongoing neuronal differentiation and synaptogenesis suggesting that this protein plays an important role in neuronal function during development and in the adult. BHLHB2 is expressed in the hippocampus, and cortex. Thus, BHLHB2 may be a critical transcription factor involved in the physiology and pathophysiology of NMDA receptor function. Low level NMDA receptor activation, which establishes a neuroprotective state in cultured neurons, was employed to determine the occupancy of two pro-survival

transcription factors, cAMP response element binding (CREB) protein and nuclear factor kappaB (NF-κB), and BHLHB2. NMDA receptor-dependent activation of promoter IV increases the binding of CREB and NF-κB while decreasing BHLHB2 occupancy. In a BHLHB2 knock-out mouse model, injection of kainic acid significantly increases exon 4-specific BDNF mRNA levels in the hippocampus. Because BHLHB2 is a hypoxia-inducible gene and is expressed in brain regions that are vulnerable to hypoxic-ischemic neuronal injury, BHLHB2 may be a new target for drug development in stroke.

p53 involvement in Alzheimer's disease: how fibroblasts may link neurons with lymphocytes

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Alzheimer's disease (AD) is the most common form of dementia among elderly people, that gradually destroys brain cells and leads to progressive decline in mental function. AD is difficult to diagnose, especially in the early course of the disease. The confirmatory diagnosis of AD is possible only post mortem and is based on the recognition and quantification of senile plaques and neurofibrillary tangles. Detection of early disease-related biomarkers is crucial to facilitate the development of new diagnostic tools and drug therapies. In the search of putative biomarkers, an intriguing correlation between p53 and AD has been recently demonstrated. Based on previous work on fibroblasts, we demonstrated that peripheral blood cells from sporadic AD patients specifically express an anomalous and detectable conformational state of p53 (mutant p53) that allows to differentiate them from peripheral blood cells of age-matched non-AD subjects. In this regard, peripheral changes of the immune system have been reported including lymphocytes function and subset distribution. We hypothesize that the pathological processes leading to AD would cause characteristic changes in circulating peripheral cells, generating a detectable disease-specific cell phenotypes. In this contest, lymphocytes appear to be the most interesting cells to be analyzed. The relevance of this hypothesis is twice. From one side, the presence of peripheral markers of AD may represent novel putative diagnostic tool to improve a clinical diagnosis of AD. From the other, AD should be considered as a systemic disease.

Enhancement of domoic acid toxicity by polyether compounds related to clupeotoxism

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Clupeotoxism, a human syndrome due to clupeoid fish poisoning that may include neurological problems and death, has been originally associated with the polyether molecule palytoxin. The toxic action of palytoxin has been attributed to its capability to transform the Na^+/K^+ pump in a non selective cation channel, inducing a progressive depolarization of cell membrane potential and ultimately cell death. We have used cultured cerebellar neurons to determinate the neurotoxicity of palytoxin and to explore the possibility of interaction between palytoxin and the amnesic seafood excitotoxin domoic acid, which toxicity has been already characterized by our group in these neurons. Exposure of neuronal cultures to high picomolar concentrations of palytoxin caused a slow neurodegeneration characterized by initial swelling of the cell bodies and followed by both neurite and cell body degeneration after 24 h. Only 10% neuronal survival was observed 24 h after exposure to 500 pM palytoxin. Intracellular Ca^{2+} concentration following 2 h exposure to palytoxin increased 2–3 times over basal levels. Exposure of cultures to subtoxic concentrations of both palytoxin and domoic acid resulted in massive neurodegeneration without any further increase in intracellular Ca^{2+} concentration. The synergism between palytoxin and domoic acid in producing neurodegeneration was prevented by the non-NMDA receptor antagonist CNQX, but neither by NMDA receptor antagonists nor VSCC blockers.

These results provide further insight on the excitotoxic process and suggest the need to establish new safety limits for the presence of palytoxin and domoic acid in seafood.

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Behavioural and electrographic evidence of seizure reduction after pharmacological preconditioning with domoic acid

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Previously we have shown that low-dose domoic acid (DA) preconditioning produces tolerance to the behavioural effects of high-dose DA. In the present study we used electrocorticography (ECoG) to monitor subtle CNS changes during and after preconditioning. Young adult male Sprague Dawley rats were implanted with a left cortical electrode and acute cortical recordings were obtained during preconditioning by intrahippocampal administration of either low-dose domoic acid (15 pmole) or saline, followed by a high-dose DA (100 pmole) challenge. The ECoG data were analysed using fast Fourier transformation to obtain the percentage of baseline power spectral density for low and high frequency ranges (delta-gamma; range: 1.25–100 Hz). Consistent with previous results, behavioural analysis confirmed that low-dose DA preconditioning 60 min before a high-dose DA challenge produced significant reductions in cumulative seizure scores and high level seizure behaviours. ECoG analysis revealed significant reductions in both power spectral density across delta-beta frequency bands (delta, $P < 0.0001$; theta, $P < 0.0037$; alpha, $P < 0.002$; beta, $P < 0.002$) and high frequency/high amplitude spiking in DA preconditioned animals, relative to saline controls. Significant correlations between seizure scores and cortical ECoG power confirmed that behavioural analysis is a reliable marker for seizure analysis. The reduction of power in delta to beta frequency bands in contralateral cortex does not allow a clear distinction between seizure initiation and seizure propagation, but does provide objective confirmation that pharmacological preconditioning by DA reduces network seizure activity.

$GABA_C$ receptor mechanisms in the rat amygdala and their role in the modulation of fear and anxiety

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It is generally accepted that the amygdala plays a prominent role in modulating anxiety. Moreover, GABA neurons are widely distributed in the amygdala and particularly within the main intercalated and paracapsular islands, and the lateral division of the central nucleus (CeA) where they seem to form an inhibitory interface that may control the trafficking of nerve impulses from the amygdaloid basolateral complex and other brain regions to the medial CeA. As projections from the latter amygdaloid region to specific hypothalamic and autonomic brain stem nuclei give rise to different signs of fear and anxiety GABA neurons appear to be in a crucial position in the network to modulate these responses. The $GABA_C$ receptor is a new member of the ionotropic receptor family which differs from the $GABA_A$ receptor because it lacks sensitivity to bicuculine and forms functional homomeric receptors by the assembly of ρ subunits. $GABA_C$ receptors have much higher affinity for GABA and a slower rate of desensitization than the $GABA_A$ receptors and are thereby ideally suited to exert tonic inhibitory actions. Since $GABA_C$ receptors have been found in some selected brain regions and there exists electrophysiological evidence suggesting the presence of these receptors within the lateral CeA, it was of great interest to perform behavioural experiments aimed at exploring simultaneously both their existence within the amygdala and their possible role in the modulation of fear and anxiety. For this purpose, the selective $GABA_C$ antagonist 1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) was infused bilaterally near the lateral CeA and the intercalated paracapsular islands, and the behavior was studied in the Elevated Plus-Maze. For the sake of comparison bicuculline was infused under the same experimental conditions in a different set of experiments. It was found that TPMPA at a low dose range (6–240 pmole/side), but not at a higher dose (800 pmol/side) decreased in a dose dependent way the time spent by the rats in the open arms of the maze as compared to the saline treated controls. Opposite effects were observed on the exploration of the closed arms of the maze. No effects of the administration of TPMPA were observed in the number of visits to the open arms of the maze, and no convulsions developed at any of the doses used. Bicuculline (1.8–60 pmol/side), unlike TPMPA had no effects on the exploration of the maze. The appearance of convulsions precluded the infusion of higher bicuculline doses. No effects of TPMPA or bicuculline were observed on the locomotion of the rats; both in the elevated plus-maze and an open-field. Our results support the notion that $GABA_C$ receptors do exist in the rat amygdala and that they modulate fear and anxiety by exerting a tonic inhibitory effect on the anxiogenic output of the amygdala.

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Protecting against seizure-induced neuropathology with GluR5KR antagonists

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Organophosphorus compounds are potent neurotoxic chemicals that are widely used in industry and agriculture. Employed as insecticides, worldwide, they are yearly responsible of several millions of poisonings. Nerve agents, which are the most toxic of the chemical warfare agents, are also organophosphorus compounds. Presently available countermeasures against nerve agents are not adequately effective in preventing brain injury, which is caused primarily by intense seizure activity. Profound brain damage due to nerve agent exposure can cause death, or long-term, severe neurological and behavioral illnesses. Therefore, there is an urgent need for development of safe and effective antidotes that will stop or reduce seizures and prevent brain damage after exposure. We have developed an in vitro model of rat brain slices containing both the amygdala and the hippocampus, two brain regions that are most prone to generating and spreading seizure activity. Simultaneous electrophysiological recordings from the amygdala and the hippocampus in these slices have revealed that in response to the nerve agent soman, the amygdala generates prolonged neuronal discharges resembling brain seizures, whereas the hippocampus generates only interictal-like spikes. As kainate receptors containing the GluR5 subunit play a significant role in the regulation of neuronal excitability in the amygdala and the hippocampus, and GluR5 antagonists have been shown to block epileptiform activity in other models of seizure induction, we have tested the effectiveness of these compounds against epileptiform activity induced by soman. The GluR5 antagonists UBP302 and LY293558 reduced the frequency and duration, or completely eliminated the seizure-like discharges in the amygdala, and reduced the amplitude of the interictal-like spikes in the hippocampus. *In vivo* administrastion of these GluR5 antagonists to rats exposed to soman, at 60 min after the initiation of seizures, significantly reduced brain seizures and protected against neuronal loss and degeneration, as determined using design-based stereology on Nissl-stained sections and Fluoro-Jade-C staining, respectively. Screening for pharmacological efficacy of potential neuroptotective/anticonvulsant compounds in the amygdala and the hippocampus, simultaneously, in vitro, followed by in vivo testing—when the in vitro results are promising—is a strategy that can provide reliable data in an efficient manner.

Long-term consequences of altered glutamatergic signalling during neonatal development

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We have previously reported that systemic injections of very low (subconvulsive) doses of the excitatory amino acid domoic acid (DOM)

during the second postnatal week of life in the rat, results in a reproducible behavioural syndrome with striking similarities to partial complex epilepsy when animals are exposed to novel environments as adults. Further, we have described evidence of hippocampal plasticity (mossy fibre sprouting) and elevations of some neurotrophin signaling systems in this model. We report herein that adult rats treated with DOM as neonates show reductions in both chemically and electrically-induced seizure threshold in vivo accompanied by region-selective reductions in subpopulations of GABAergic neurons in the hippocampus. Further we have recorded field potentials in the presence or absence of glutamatergic and GABAergic antagonists in isolated hippocampal slices from treated rats and found evidence of alterations in the electrical gating characteristics of the dentate gyrus. These new data argue that relatively minor changes in glutamatergic signaling during neonatal development can result in permanent changes in the balance of excitatory/inhibitory tone in the hippocampal formation, with consequent effects on behaviour and brain pathology.

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Acute and long-term neuronal loss induced by perinatal asphyxia: activation of pro-apoptotic, anti-apoptotic and sentinel proteins

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Delivery is a risky event for the newborn. The mother-dependent respiration has to be replaced by an autonomous pulmonary breathing immediately after delivery. If delayed, hypoxia is sustained, leading inevitably to cell death, even after resuscitation. Re-oxygenation triggers a metabolic cascade worsening and/or delaying the recovery of the newborn. With an experimental model in rat, we observed that perinatal asphyxia (PA) leads to neuronal death and neurite atrophy in basal ganglia, and hippocampus, with behavioural deficits reflecting the severity of the insult. Cell death is observed even 30 days after birth with apoptotic features. Pro (BAD)- and anti (BCL-2, ERK-2)-apoptotic, as well as neurogenesis and neuritogenesis (BDNF, bFGF) promoting proteins are concomitantly increased.

Nicotinamide can prevent some of the effects produced by PA, suggesting the involvement of the sentinel protein poly(ADP-ribose) polymerase-1 (PARP-1), known to be overactivated whenever there is a menace to the genome. We report here on the effect of PA on PARP-1 mRNA levels using Real Time PCR, as well as on PARP-1 activity in the nuclear fraction of brain, and heart tissue, using a colorimetric method. It was found that the expression and activity of PARP-1 was increased in brain tissue following PA. In heart, PARP-1 activity was first increased 8 h, but then decreased 24 h after delivery in PA, as compared to control pups.

In summary, the present results support the idea that PARP-1 overactivation is an underlying factor of the long term effects produced by PA, and that PARP-1 inhibition provides neuroprotection.

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Role of arginine dysregulation and elevated arginase activity in pulmonary disorders

Asthma and the role of arginase in animal models

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After almost two decades of intense scientific interest in the role of nitric oxide (NO) as an endogenous mediator of a variety of physiological and pathophysiological processes, there is now growing interest in the potential roles of arginase—as a regulator of the synthesis of NO, and of polyamines and L-proline. Recent evidence indicates that increased arginase activity and altered L-arginine homeostasis are major factors in the pathology of allergic asthma. In guinea pigs, it has been discovered that arginase activity is involved in the regulation of airway function and responsiveness by attenuating bronchodilating NO production and that increased activity of the enzyme underlies the development of allergen-induced airway hyperresponsiveness. Increased expression and activity of arginase has been confirmed in numerous other animal models of asthma and in asthmatic patients. Some of these studies have indicated that genes encoding for arginases I and II belong to the most predominantly overexpressed genes in asthma, which may be induced by Th2 cytokines implicated in the disease. By in vivo studies using a guinea pig model of allergic asthma, we have recently demonstrated that specific arginase inhibitors may effectively inhibit allergen-induced early and late asthmatic reactions, AHR after these reactions and airway inflammation. Moreover, we now demonstrate that arginase inhibition also inhibits airway remodelling in chronic asthma, which might involve NO-dependent as well as NO-independent processes, via inhibition of L-ornithine production and subsequent synthesis of polyamines and L-proline. These studies indicate that arginase inhibitors have therapeutic potential in allergic asthma.

Arginine metabolism and airflow obstruction in asthma

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Arginine metabolism has been noted to play a role in various pulmonary diseases, including asthma, through a complex interplay

between arginase and nitric oxide synthase (NOS) activity. Nitric oxide (NO) is produced from arginine via NOS. Exhaled NO levels are higher in asthmatics compared with controls and correlate with inflammatory characteristics in the airways as well as airway obstruction. However, NO also has bronchodilatory properties. Arginine can also be metabolized by arginase, forming ornithine, a precursor to polyamines and L-proline, which may promote cell proliferation and collagen production. Results from a large cohort established that single nucleotide polymorphisms for arginase I and II were associated with an increased relative risk of atopy and asthma. Another study demonstrated high arginase activity during asthma exacerbations which may reduce with treatment. Also, a recent cross-sectional study of stable severe and non-severe asthmatics and controls evaluated whether assessment of arginine bioavailability and alterations in arginine metabolism may be useful in the phenotypic characterization of asthma. In this study arginase activity and arginine availability (measured by systemic arginine concentrations respective to ornithine citrulline and methylarginines) was higher in asthmatics. Particularly in severe asthmatics, arginase activity was associated with lower lung function and worse airway obstruction, providing insight into the metabolic mechanisms that may lead to airway remodeling and obstruction in severe asthma. Therefore arginine metabolism and catabolism is a complex process, and appears to be variably affected in asthma, with differences according to clinical phenotypes.

L-arginine metabolism and arginase in cystic fibrosis lung disease

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Nitric oxide (NO) is recognized to be involved in multiple aspects of lung biology in cystic fibrosis (CF), and is known to play a key role in bronchomotor control and antimicrobial host defense. L-arginine is the common substrate for both the NO synthases and arginase families of isozymes and CF airways are deficient in NO synthesis. However, CF patients have increased airway arginase activity. Reduced L-arginine availability for NO synthesis may result in airway obstruction and impaired killing of *P. aeruginosa* in the CF lung, mechanisms which may be counteracted by increasing L-arginine availability for NO synthases in the CF lung.

Experiments in a CF mouse model using a LC-tandem spectroscopy based assay after infusion of stable amino acid isotopes show abnormalities in pulmonary L-arginine metabolism in the CF mice with up-regulated NOS and arginase activity in vivo. In addition, preliminary data from infected mice show that chronic *Pseudomonas aeruginosa* infection of the lung results in a significant alteration in L-arginine metabolism, specifically enrichment of ornithine, which matches our results from arginases activity measurements.

In regards to clinical studies we have recently shown that a single inhalation of nebulized L-arginine resulted in a significant increase of airway NO formation and pulmonary function in patients with CF (Grasemann H et al., AJRCCM 2006). We are currently conducting a double-blind, randomized, placebo-controlled crossover treatment trial in 20 CF patients to study the effects of 14 days of L-arginine inhalation on airway NO formation, pulmonary function, and inflammation. Results of this trial will be presented.

Hemolysis and arginine dysregulation

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Secondary pulmonary hypertension (PH) is emerging as one of the leading causes of mortality and morbidity in patients with hemolytic anemias such as sickle cell disease (SCD) and thalassemia. Impaired nitric oxide (NO) bioavailability represents the central feature of endothelial dysfunction, and is a major factor in the pathophysiology of PH. Inactivation of NO correlates with hemolytic rate and is associated with the erythrocyte release of cell-free hemoglobin, which consumes NO directly, and the simultaneous release of the arginine-metabolizing enzyme arginase, which limits bioavailability of the NO synthase substrate arginine during the process of intravascular hemolysis. Rapid consumption of NO is accelerated by oxygen radicals that exist in both SCD and thalassemia. A dysregulation of arginine metabolism contributes to endothelial dysfunction and PH in SCD, and is strongly associated with prospective patient mortality. The central mechanism responsible for this metabolic disorder is enhanced arginine turnover, occurring secondary to enhanced plasma arginase activity. This is consistent with a growing appreciation of the role of excessive arginase activity in human diseases, including asthma and pulmonary arterial hypertension. New treatments aimed at improving arginine and NO bioavailability through arginase inhibition, suppression of hemolytic rate, oral arginine supplementation, or use of NO donors represent potential therapeutic strategies for this common pulmonary complication of hemolytic disorders.

Arginine metabolism and transport in pulmonary hypertension

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Pulmonary hypertension (PH) is a disease characterized by vasoconstriction, vascular remodeling and low endogenous production of the potent vasodilator nitric oxide (NO) in the pulmonary circulation. This has lead to the use of exogenous NO given as an inhaled gas as a therapy for PH. However, inhaled NO has side-effects and many patients do not respond to inhaled NO, for example $\sim$40% of patients with neonatal PH do not respond to inhaled NO. Therefore, new therapeutic modalities designed to increase endogenous NO production are needed for PH. L-arginine is the substrate for the NO synthases (NOS) which produce NO and L-citrulline. The transport of L-arginine into endothelial cells depends in large part on the cationic amino acid transporters (CAT). Alterations in CAT-mediated L-arginine transport affect NO production in the lung, for example adding L-arginine increases NO production, while inhibiting L-arginine uptake by CAT decreases NO production. Indeed, the expression of CAT is altered in hypoxia (a model of PH), and in this setting NO production can be augmented by over-expressing CAT in endothelial cells. Once L-arginine is transported into endothelial cells it can also be metabolized by arginase to produce urea and L-ornithine. Indeed, inhibiting arginase activity augments lung NO production in endothelial cells. Thus, L-arginine transport by CAT and L-arginine metabolism by arginase are two key regulators of lung NO production and represent novel therapeutic targets to augment NO production in PH.

Arterial injury induces L-arginine metabolizing enzymes to promote neointima formation

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Proliferation of vascular smooth muscle cells (VSMCs) plays a critical role in the formation of neointimal lesions; however, the mechanisms responsible for neointima development are not fully known. Since L-arginine is metabolized to important growth regulatory molecules, the present study investigated whether L-arginine metabolizing enzymes contribute to neointima formation following arterial injury. Balloon injury of rat carotid arteries resulted in a rapid increase in inducible nitric oxide synthase (iNOS), arginase I, and ornithine decarboxylase (ODC) expression. ODC activity peaked 1 day after arterial damage while arginase I and iNOS activity peaked 7 days following injury. The increase in arginase activity was associated with an approximate two-fold increase in arginase I protein expression in the vessel wall. Immunohistochemistry detected minimal arginase I staining in uninjured carotid arteries; however, 7 days after injury arginase I staining was observed throughout the vessel wall but intense arginase I expression was detected in the neointima. Local perivascular application of the arginase inhibitors (*S*)-2-boronoethyl-L-cysteine or *N*-hydroxy-nor-L-arginine immediately after injury markedly attenuated VSMC proliferation and neointima formation 14 days after injury. In contrast, the arginase inhibitors had no effect on VSMC apoptosis or endothelial cell regrowth following arterial injury. Finally, the local application of the ODC inhibitor α-difluoromethylornithine also inhibited neointima formation. In conclusion these results demonstrate that arterial injury induces the expression of iNOS, arginase I, and ODC, and that local application of arginase or ODC inhibitors block intimal thickening. These results identify L-arginine metabolic pathways as attractive therapeutic targets in treating occlusive vascular disease.

Role of fibrinogen in microcirculation

Fibrinogen variants and association with risk of cardiovascular disease

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Plasma fibrinogen levels have consistently been associated with risk of cardiovascular disease. Fibrinogen is a very heterogenous molecule and many common variants have been described. Common fibrinogen variants include the low molecular weight form (LMW-fibrinogen, MW 305 kDa, ±26% of total fibrinogen) and the LMW' form (MW 270 kDa, ±4% of total fibrinogen) which are the result of partial degradation of one or two Aα-chains. In the αC domain the coagulation rate, lateral aggregation, binding to integrins is located. Another common variant is the γ' form of fibrinogen (7–15%) which is the result of alternative splicing of the fibrinogen γ-chain. This variant of fibrinogen has increased thrombin and FXIII binding, while it lacks the platelet binding site. The fibrinogen genes contain a large number of polymorphisms, of which several have been shown to affect fibrinogen levels or fibrinogen functions. Since each form has its own specific characteristics on clotting rate, fibrin network structure or fibrinolysis, it may be expected that these fibrinogen variants are differently associated with risk of cardiovascular disease. In the presentation, an overview will be presented on the associations, and the possible underlying mechanisms, between fibrinogen variants and cardiovascular disease.

Vessel-specific vasodilation by fibrinogen: role of the $\alpha_v ß_3$ integrin v

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Human fibrinogen is a circulating 340 kDa glycoprotein, primarily synthesised by hepatocytes with a half-life of around 100 h. The normal plasma concentration ranges between 4.8 and 10.5 μM (160–350 mg/dl). During episodes of inflammation, the synthesis of fibrinogen is increased, an effect thought to be mediated by interleukin-6 (IL-6). Contrary to the well-known importance in coagulation and the acceleration on cardiovascular disease progression, little is known about direct vasomotor effects of fibrinogen. Both vasodilation and vasoconstriction have been reported.

We aimed to examine a possible contribution of fibrinogen to the regulation of vascular tone by investigating the vasomotor effects of fibrinogen over the full range of plasma concentrations. Furthermore, the effect of fibrinogen on bradykinin-induced vasodilation was explored. We used purified human fibrinogen, purified bovine fibrinogen and crude commercially available bovine fibrinogen. Rings of human mammary artery (IMA), small porcine coronary (SPCA) and porcine tongue arteries (PTA) were prepared and investigated in-vitro. Primary cultures of porcine aortic endothelial cells (PAEC) and human umbilical vein endothelial cells (HUVEC) were established to investigate the effects of fibrinogen on the expression of the bradykinin receptor type 2 (BKR-2) which is constitutively expressed in endothelial cells.

Fibrinogen appears to be a vessel-specific vasodilator in blood vessels with comparably high expression of the $\alpha_v\beta_3$ integrin. In these blood vessels fibrinogen also potentiates vasodilation by bradykinin. While fibrinogen increases the activation of the vascular NO/cGMP pathway stimulated by bradykinin, it has no effect on the constitutive expression of BKR-2 in endothelial cells.

Direct effects of fibrinogen on human smooth muscle cell phenotype

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Fibrinogen is deposited in the vessel wall at atherosclerosis-prone sites and increased fibrinogen levels are considered as an independent atherogenic risk factor. Fibrinogen directly promotes proliferation and migration of smooth muscle cells (SMC) and may thereby contribute to vascular lesion formation. In addition, interactions of fibrinogen with the intercellular adhesion molecule-1 (ICAM-1) mediate cellular adhesion and trans-endothelial leukocyte invasion. In the present study,

we demonstrate that fibrinogen-induced migration of human vascular SMC in mediated by binding to ICAM-1 which results in activation of the Akt and p38-MAPK pathway. SMC were explanted from human saphenous veins and cultured in media with 10% fetal calf serum. Fibrinogen concentration-dependently (1–100 μM) induced SMC migration in Boyden chamber assays. Phosphorylation of extracellular-regulated kinases (ERK 1/2), Akt, and p38 in fibrinogen-stimulated SMC was determined by Western blotting. Fibrinogen-induced cell migration was inhibited by antibodies to ICAM-1 (10 μg/ml), the PI3-kinase inhibitor LY294002 (10 μM) and the p38 inhibitor SB203580 (10 μM). In contrast, the MEK inhibitor PD98059 (10 μM) and the GPIIb/IIIa antagonist abciximab (10 μg/ml) had no effect on SMC migration. In line with these observations, ICAM-1 antibodies, but not abxicimab, inhibited fibrinogen-induced phosphorylation of Akt and p38. Fibrin-induced phosphorylation of ERK 1/2 was not affected by ICAM-1 antibodies or by abxicimab. These data suggest that fibrinogen stimulates migration of human vascular SMC via binding to ICAM-1 and activation of Akt and p38. Our study clarifies the role of ICAM-1 for fibrinogen-induced SMC chemotaxis which may contribute to the proatherogenic effects of fibrinogen.

Fibrinogen-induced endothelial cell layer permeability, role of MMP

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Fibrinogen (Fg) is a well known inflammatory agent. The slightest elevation of its plasma content increases risk of cardiovascular complications associated with diseases such as hypertension, diabetes, and stroke. Recent studies have shown that Fg exhibits vasoactive properties and alters endothelial cell (EC) layer integrity. We found that Fg binding to endothelial intercellular adhesion molecule 1 (ICAM-1) causes arteriolar constriction in vitro and *in vivo*. Our recent studies show that Fg may have a role in the regulatory production of the most potent vasoconstrictor endothelin-1 (ET-1) from ECs. Recently we found that elevated content of Fg increases permeability of EC layer to albumin through formation of filamentous actin (F-actin). Fg itself, although it takes a longer time, can leak through the EC layer. Subsequent studies suggested that increased binding of Fg to EC alters expression of tight junction proteins through extracellular signal-regulated kinases-1 and 2 (ERK-1/2) signaling and adherence junction proteins through activation of matrix metalloproteinases. Combined, these results indicate that Fg may have a significant role in microcirculatory complications arising during diseases such as hypertension, diabetes, and stroke, which are typically accompanied with increased content of this plasma adhesion protein.

Roles of glutamatergic transmission and plasticity in pain

Spinal AMPA receptor plasticity in inflammatory pain

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Pain hypersensitivity accompanying inflammation or tissue injury is a physiological response that serves to protect the injured tissue from adverse behaviour. However, sometimes this hypersensitivity outlasts the initial injury or is the result of injury to the nervous system, in which case it becomes pathological. It has long been known that central mechanisms contribute to pain hypersensitivity, and evidence suggest that LTP-like potentiation of nociceptive primary afferent synapses may be one such mechanism. However, both the molecular mechanisms that underlie such plasticity and the specific synaptic populations involved are unclear. Using neuronal tracing and immunoelectron microscopy, we have shown that the capsaicin model of inflammatory pain is associated with an elevated density of GluA1- but not GluA2/3-containing AMPA-type glutamate receptors at synapses formed by C-fibers that lack neuropeptides such as substance P. By contrast, little or no change in AMPA receptor subunits occur at synapses from nociceptive primary afferent fibers expressing substance P. Curiously, autophosphorylated CaMKII, which is strongly implicated in synaptic plasticity at central glutamatergic synapses, shows decreased levels in the postsynaptic density of synapses from substance P-lacking C-fibers but increased levels postsynaptic to substance P-containing nociceptors in the same inflammatory pain model. Thus, potentiation of non-peptidergic C-fiber synapses by recruitment of GluA1-containing, possibly Ca^{2+}-permeable AMPA receptors may underlie some aspects of inflammatory pain.

Furthermore, unlike in proposed LTP mechanisms at other glutamatergic synapses, there appears to be a dissociation between CaMKII autophosphorylation and activity-dependent AMPA receptor trafficking at the two major types of nociceptive primary afferent synapse.

Novel forms of long-term potentiation at spinal synapses of nociceptive C-fibers

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Activity-dependent forms of long-term potentiation (LTP) at glutamatergic synapses in the hippocampus may underlie learning and memory formation. We identified LTP at spinal synapses of nociceptive C-fibre afferents that may cause pain amplification. LTP was induced by either high frequency (100 Hz) or by low frequency (2 Hz) conditioning electrical nerve stimulation or by natural noxious stimuli or acute nerve injury. These different forms of spinal LTP can be induced in vivo and in vitro, involve distinct populations of nociceptive spinal dorsal horn projection neurons and require Ca^{2+}-dependent signalling pathways. In addition, LTP was also induced in the absence of any presynaptic activity upon abrupt but not upon tapered withdrawal from brief (60 min) application μ-opioid receptor agonists. Postsynaptic G-protein coupling was required for induction of opioidergic LTP upon withdrawal from opioids, in contrast to the acute synaptic depression during the application of the opioid. Some nociceptive dorsal horn neurons responded with a Ca^{2+} rise to the application and/or the withdrawal of the opioid in vitro or in vivo. The Ca^{2+} rise upon withdrawal from the opioid was indispensable for the induction of opioidergic LTP. These various forms of LTP at spinal nociceptive synapses may contribute to pain amplification (hyperalgesia) after peripheral trauma, nerve injury or inflammation and to opioid-induced hyperalgesia.

Cortical plasticity as a cellular model for chronic pain

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Investigation of molecular and cellular mechanisms of synaptic plasticity is the major focus of many neuroscientists. There are two major reasons for searching new genes and molecules contributing to central plasticity: first, it provides basic neural mechanism for learning and memory, a key function of the brain; second, it provides new targets for treating brain-related disease. Here, I propose that LTP in the anterior cingulate cortex (ACC) as a synaptic model for chronic pain in the brain. Integrative approaches including genetic, neurobiological and physiological methods are used to investigate the roles of cortical neurons and microglia in pain-related synaptic LTP and chronic pain. We found that both presynaptic enhancement of glutamate release and postsynaptic potentiation of AMPA receptors contribute to chronic pain such as inflammatory pain and neuropathic pain. Furthermore, calcium-stimulated adenylyl cyclase subtype 1 (AC1) is critical for triggering both presynaptic and postsynaptic changes. By contrast, microglia only contributes to changes in spinal dorsal horn, but not in the cortex. Our findings strongly suggest that e that ACC LTP may serve as a cellular model for studying central sensitization that related to chronic pain, as well as pain-related cognitive emotional disorders.

A behavioural fish model of nociception for testing new analgesic drugs

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Research on pain and analgesia has contributed significantly to better treatment strategies for human pain-related disorders. As the goal is to develop safer and more effective pain treatments for patients, mainly mammalian models are used to test analgesic drugs by inducing a "painful" state in the animal via a noxious stimulus. However, from an ethical point of view, this is a controversial issue, and encourages the development of alternative models. Evidences suggest that vertebrate fish can respond to nociceptive stimuli and that they possess the necessary sensory components to detect and respond to pain similarly to those of higher vertebrates. Especially Zebrafish (*Danio rerio*) is considered as an alternative model species, mainly due to its simple nervous system, well studied genetics, behaviour, and amenability for testing purposes. We have developed an acute pain model based upon the behavioural responses of zebrafish to acetic acid. This compound was chosen because its protons are known to stimulate nociceptive receptors. Time–course behavioural changes were investigated before and after the injection to the lips of fish, and locomotory activity of housed individuals were automatically recorded by a biomonitor system. We observed nociceptive-related behaviour patterns in response to acid acetic, i.e. an increased lethargy and respiratory frequency, which lasted for over 90 min. This confirms nociception in zebrafish and shows that pain-related responses in fish can be used for testing analgesic drugs. Currently, we are validating the model by investigating the inhibition of reference analgesic drugs (morphine and ibuprofen) on the nociceptive behaviours.

Intrathecally injected Ile-Pro-Ile, an inhibitor of membrane ectoenzyme dipeptidyl peptidase IV (CD26, EC 3.4.14.5), is antihyperalgesic in rats by switching the enzyme from hydrolase to synthase functional mode to generate endomorphin 2

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Background: We have found recently that membrane-bound dipeptidyl peptidase IV (DPP-IV) generated extracellularly immunoreactive endomorphin 2 (E2) from Tyr-Pro precursor in a depolarisation-sensitive manner in rat isolated L4,5 dorsal root ganglia when the enzyme was switched to synthase mode by the hydrolase inhibitor Ile-Pro-Ile (IPI)(presentation by Rónai et al., this Congress).
Results: We induced hyperalgesia in rats by injecting carrageenan into the right hindpaw and measured the reduction in pain threshold to pressure (Randall–Selitto test). The hyperalgesia, peaking at 180 min after injection, was fully reversed by *intrathecal (it)* administration of 30 nmol/rat IPI. The antihyperalgesic action was antagonized by *sc* naloxone (1 mg/kg) and *it* injected specific antiserum to E2 indicating that the opioid receptor-mediated effect was produced by an endogenously generated E2-like immunoreactive substance. *Intrathecal* IPI was ineffective as analgesic in acute pain perception test such as rat tail-flick, whereas E2 ($EC_{50} = 13.3$ nmol/rat), E1 (6.8 nmol/rat), morphine (0.11 nmol/rat) and DAMGO (0.0059 nmol/rat) exerted opioid receptor-mediated analgesia given by the same route.
Conclusion: Carrageenan-induced C-fiber barrage ("wind-up") may create ideal conditions for the de novo synthesis of E2 in rat spinal cord dorsal horn if the enzyme DPP-IV is switched to "synthase" functional mode by IPI.

Synthesis and structure-activity relationships of novel hexapeptides with Cα,α- disubstituted cyclic amino phosphonates as nociceptin receptor ligands

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Nociceptin/Orphanin FQ (N/OFQ) is a heptadecapeptide, endogenous ligand for the G(i)-protein-coupled N/OFQ receptor (NOP), structurally and functionally related to the classical opioid receptors. The N/OFQ and its NOP receptor modulate a variety of biological functions, both at central and peripheral level. The nociceptin receptor is

activated also by non-peptide agonists and by short 6-amino acid peptides as exemplified by Ac-RYYRWK-NH_2, which was identified from a large combinatorial library of hexapeptides as a very potent partial agonist for mouse NOP. Ac-RYYRWK-NH_2 has been radiolabelled and characterised in receptor-binding experiments. [^{3}H]Ac-RYYRWK-NH_2 is shown to be a selective ligand for the nociceptin receptor and may be useful for further understanding of ligand-binding domain of this receptor. The hexapeptides are used as chemical templates in structure-activity relationships (SAR) studies for identification of novel NOP receptor ligands. Based on the template Ac-RYYRWK-NH_2, new hexapeptides analogues have been synthesised with Cα,α-disubstituted cyclic amino phosphonates at position 1, using SPPS Fmoc-chemistry.

The biological activity of the newly synthesized peptides has been tested on electrically stimulated preparations isolated from rat vas deferens and compared to Ac-RYYRWK-NH_2, discussing the results in relation to structure–activity relationships.

S100 proteins in health and disease

Insights into the function of S100B from structural and spectroscopic studies

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S100B is a member of the S100 protein family which constitutes the largest subfamily of Ca^{2+}-binding EF-hand proteins in human. It is ubiquitously expressed in all tissues, including the human brain where it is present in very high amounts. Remarkably, S100B has both intra- and extracellular functions. Intracellularly S100B transduces Ca^{2+} signals, whereas it acts extracellularly as a neurotrophic and neuroprotective cytokine via the Receptor for Advanced Glycation Endproducts (RAGE). Besides Ca^{2+}, S100B binds other divalent metal ions like Zn^{2+} and Cu^{2+} with high affinity; however the precise function of Zn^{2+} and Cu^{2+} remains still unknown. We are trying to understand these multiple functions of S100B at a molecular level applying X-ray crystallography and spectroscopic techniques like surface plasmon resonance (SPR), electron paramagnetic resonance (EPR) and UV-visible spectroscopy. We could show recently that S100B occurs as multimers in the brain which bind with higher affinity to RAGE inducing stronger activation of cell growth and survival. Moreover, SPR measurements revealed that Zn^{2+}-binding increases as well S100B's affinity towards RAGE implying an extracellular regulatory role of Zn^{2+}. EPR studies on Cu^{2+}-binding to S100B demonstrated that Cu^{2+} competes with Zn^{2+} for the same binding site in S100B but binds with several fold affinity and leads to oxidative modifications of S100B.

Posttranslational modifications of the metastasis-promoting protein S100A4

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Elevated S100A4 levels are associated with the metastatic phenotype of tumor cells and with adverse prognosis in several tumor types. Although the basis for its metastasis-promoting effects is incompletely understood, S100A4 has been linked to multiple cellular events of known relevance to the metastatic process, such as invasion and angiogenesis. S100A4 has been identified extracellularly, in the cytoplasm and in the nucleus of tumor cells, but the biological implications of subcellular localization are unknown. Associations between a variety of posttranslational protein modifications and altered biological functions of proteins are becoming increasingly evident, and such alterations may have an impact on the metastatic process. Several S100 proteins are reported to be posttranslationally modified with known biological implications. Identification and characterization of posttranslationally modified S100A4 variants could thus contribute to elucidating the mechanisms for the many cellular functions that have been reported for this protein. Using in-house produced antibodies we immunoprecipitated S100A4 from protein lysates from colorectal carcinomas and cell lines. When resolved by 2-dimensional PAGE and visualized by western immunoblotting, S100A4 from whole cell lysates and immunoprecipitates showed a characteristic pattern of numerous horizontally aligned spots, suggesting the presence of several charge variants. Several of these have been further verified to be S100A4 by mass spectrometry, and our findings indicate the existence of posttranslational modifications of S100A4. Similar, although not identical, findings have been reported by other groups. Results from our studies towards the nature of these posttranslational modifications and their possible biological implications will be presented.

Does S100A6 (calcyclin) influence the function of CacyBP/SIP and Sgt1?

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S100A6 (calcyclin) is a low molecular weight calcium binding protein belonging to the S100 family. High level of S100A6 has been found in fibroblasts, epithelial cells and in tumor cells with high metastatic activity. S100A6, similarly to other Ca^{2+}-sensors, changes its conformation after binding of calcium ions and interacts with several target proteins such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), annexin II, annexin VI and annexin XI. S100A6 might also interact with membrane proteins since it was found to be associated with prolactin receptor and RAGE. The chicken isoform of S100A6 binds caldesmon, tropomyosin, calponin and lysozyme. The interactions between S100A6 and these targets occurred at high concentration of Ca^{2+} and their physiological role is unclear. The search for new targets of S100A6 led to the identification of CacyBP/SIP and of Sgt1 proteins. Both CacyBP/SIP and Sgt1, interact, besides S100 proteins, with other ligands. For instance, it has been shown that CacyBP/SIP binds tubulin and ERK1/2 and through these interactions might play a role in differentiation of neuroblastoma NB2a cells. A CacyBP/SIP homolog, the Sgt1 protein, binds to some heat shock proteins such as Hsp90 or Hsp70. The results obtained recently in our laboratory indicate that S100A6 influences the activity of the complexes formed by CacyBP/SIP and Sgt1. Thus, in this presentation the role of S100A6 in the activity of CacyBP/SIP and Sgt1 will be discussed.

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S100A11, a dual mediator for growth regulation of normal human keratinocytes

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Epidermal keratinocytes provides a rare opportunity for studying growth regulation of normal epithelial cells of human origin. We previously demonstrated that S100C/A11 is a key mediator for growth inhibition of normal human epidermal keratinocytes (NHK) triggered by high Ca^{2+} or TGFβ. On exposure of NHK cells to either agent, S100C/A11 is transferred to nuclei, where it induces p21WAF1/CIP1 through activation of Sp1/Sp3. High Ca^{2+} activated NFAT1 through calcineurin-dependent dephosphorylation. In growing NHK cells, KLF16, a member of the Sp/KLF family, bound to the p21WAF1/CIP1 promoter and thereby inhibited the transcription of p21WAF1/CIP1. Sp1 complexed with NFAT1 in high Ca^{2+}-treated cells or with Smad3 in TGFβ1-treated cells, but not Sp1 alone, replaced KLF16 from the p21WAF1/CIP1 promoter and transcriptionally activated the p21WAF1/CIP1 gene. Furthermore, we showed that Ca^{2+}-dependent binding of S100A11 to annexin A1 facilitated the binding of the latter to cPLA2, resulting in inhibition of cPLA2 activity, which is essential for the growth of NHK. On the other hand, S100A11 has been shown to be overexpressed in many human cancers. We found that (1) S100A11 is actively secreted by NHK, (2) extracellular S100A11 acts on NHK to enhance the production of EGF family proteins, resulting in growth stimulation, (3) RAGE (receptor for advanced glycation endproducts), NFkB, Akt, and CREB are involved in the S100A11-triggered signal transduction, and (4) production and secretion of S100A11 is markedly enhanced in human squamous cancer cells. These findings indicate that S100A11 plays a dual role in growth regulation of epithelial cells.

Interactome of S100 proteins

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The studies of S100 functions were always obscured by redundancy and overlapping targets. This situation frustrates biochemists and cell biologists in the hunt for understanding the function of individual family members. It was also challenging for medical research and pharmacology to link a specific S100 protein to one specific disease or pathological condition. Rapid development of Structural and Chemical Biology and System Biology should make it possible to strongly engage S100 studies in non-hypothesis driven research, where huge amount of data from a wide range of experimental technologies could be combined and analysed resulting in discoveries that come out from the data, rather from specific experiments designed to back up or invalidate given hypotheses. It seems timely to establish an International interactome project for S100 protein family to explore their functions and therapeutic potential. These data should include a comprehensive set of S100 cellular targets, and the project should be supported by generation of S100 protein-specific renewable affinity reagents, synthetic protein binders and tools for their cellular knockdown and delivery to intra- and extracellular compartments. The focus will be made on the interactions dependent on the ability of S100 proteins to inhibit oligomerisation of other proteins with a high-potential therapeutic relevance. Some interesting features of existing and far from comprehensive S100 interactome include: prevalence of low affinity and transient interactions; ability of S100 proteins to interconnect hubs (proteins that have many other connections) and also to overlap cellular signaling pathways, i.e. interacting with non-muscle myosin, p53, growth factors, etc.

Inflammation-associated S100 proteins: new mechanisms that regulate function

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S100A8, S100A9 and S100A12 are constitutively expressed in neutrophils and induced in several cell types and are important in host defence. S100A12 is a potent mast cell (MC) activator, and monocyte and MC chemoattractant. Hydrophobic amino acids within the hinge domain ($S100A12_{38–53}$; I44A, I47A, I53A) are essential for function. Cell-surface interaction is unlikely to occur via RAGE, but via a G-protein-coupled mechanism. S100A12 did not induce cytokines in monocytes/macrophages, but strongly induced MC pro-inflammatory cytokines. S100A12 forms complexes with Zn^{2+}; these are evident in human atheroma. By chelating Zn^{2+}, S100A12 significantly inhibited MMP-2, -3 and -9, and using an antibody that specifically recognized the Zn^{2+}-in complex, this co-localized with MMP-9 in foam cells in atheroma. S100A8 is a potent oxidant scavenger; gene induction in macrophages is dependent on IL-10 and STAT-3, supporting a protective role. Mild HOCl oxidation generates intra-chain sulfinamide bonds; stronger oxidation promotes cross-linked forms. The latter are seen in human atheroma and S100A8 may protect LDL from oxidation because it is $\sim$200-fold more sensitive to oxidation. S100A8 and S100A9 are readily *S*-nitrosylated. S100A8-SNO suppressed MC activation and inflammation in the rat mesenteric microcirculation. S100A8-SNO is relatively stable and *S*-nitrosylates hemoglobin, making it a potential NO transporter that could regulate vessel tone in inflammatory lesions. In summary, S100A12 has pro-inflammatory properties that are likely stable in an oxidative environment, because of its lack of Cys and Met residues. On the other hand, S100A8 oxidation and *S*-nitrosylation may be important in resolution of inflammation.

S100B: a pluripotent regulator of cardiac remodeling in disease

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S100 proteins, a subfamily of EF-hand calcium-binding proteins, have putative roles in regulating cellular metabolism, growth, and differentiation with expression associated with human disease. S100B is a canonical member of this family with diverse, cell lineage-dependent signal transduction pathways, involving calcium-dependent binding to protein kinase substrates and intracellular effector protein targets, and cellular secretion to exert receptor-mediated extracellular effects, in part, by binding to the cell surface receptor for advanced glycation end-products (RAGE). RAGE ligands are implicated in the vascular response to injury and

diabetes mellitus. S100B basal expression is restricted to glial and neuronal cells but anomalously expressed in myocardium following myocardial infarction in humans and coronary artery ligation and hemodynamic load in rats. Forced expression of S100B in cardiac myocyte cultures and in transgenic mice, prevents the development of hypertrophy and the associated activation of "fetal" β-myosin heavy chain (MHC) and skeletal α-actin, by inhibiting α-adrenergic and protein kinase C-dependent signaling pathways. S100B induction post-infarction modulates the complex hypertrophic, apoptotic, and gene expression components of left ventricular remodeling with implications for function and survival; extracellular S100B induces dose-dependent cardiac myocyte apoptosis distinct from intracellular inhibition of PKC signaling; and intracellular S100B colocalizes with other S100 proteins in cardiac myocytes but confers distinct phenotypic consequences. Taken together these observations not only demonstrate a role for S100B in cardiac disease but also define cardiac muscle as an attractive model for the study of both physiologic consequences of S100 proteins and their mechanisms of action.

S100A7 (psoriasin) and S100A15 (Koebnerisin) are almost identical in sequence but highly distinct in function: implications for disease pathogenesis

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Overexpression of the hS100 protein of the A7 type (hS100A7, psoriasin) in inflammatory diseases such as psoriasis has functional implications for disease pathogenesis. The highly homologous hS100A15 (Koebnerisin, >90% sequence identity) was recently identified as similarly upregulated in inflamed psoriatic skin. However, the biological impact of the co-expression of these closely related genes remains undefined. By generating specific antibodies, we demonstrate that hS100A7 and hS100A15 are differentially expressed in specific cell types of human skin. Both proteins are chemoattractants but differ in their chemotactic activity towards specific leukocyte subtypes. Furthermore, hS100A7 and hS100A15 stimulate chemotaxis through activation of different classes of receptors. hS100A7 but not hS100A15 binds and directly mediates chemotaxis through the pattern recognition receptor RAGE (receptor of advanced glycated end products) in both in vitro chemotaxis assays and in vivo in mouse models. hS100A7-RAGE binding, signaling and chemotaxis are zinc-dependent in vitro, reflecting the zinc-mediated changes in the hS100A7 dimer structure. In vivo, chemotaxis is potentiated by combined treatment with hS100A7 and hS100A15. Thus, the evolutionary recent duplication of hS100A7/hS100A15 produced biological diversification by which their independent actions through distinct receptors regulate physiological functions but potentiate their proinflammatory activities in disease.

Circulating S100A12: a novel player in atherosclerosis?

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S100A12 is a member of the S100 family f EF-hand calcium-binding proteins. Besides calcium binding S100A12 also shows high affinity for zinc and copper ions. Extracellular S100A12 is predominantly secreted by granulocytes and monocytes and is part of the innate immune response. S100A12 is markedly overexpressed in inflammatory compartments, and elevated serum levels of S100A12 are found in patients suffering from various inflammatory and metabolic disorders. In this regard, binding of copper by S100A12 is assumed to play a pathogenic role. In vitro experiments show that copper-bound S100A12 can function as a pro-oxidant agent by supporting both copper reduction and copper redox-cycling, respectively. As a consequence, copper-bound S100A12 significantly enhances and accelerates oxidation of human low density lipoprotein lipids and apolipoproteins, respectively. Furthermore, copper-bound S100A12 stimulates proinflammatory activation of endothelial cells, granulocytes, and monocytes. These processes were substantially suppressed in the presence of redox-inert copper-chelating or radical-scavenging agents. Clinical examinations show significantly elevated plasma S100A12 levels in subjects with impaired glucose tolerance, newly-diagnosed diabetes mellitus Type 2, and acute rheumatoid arthritis (1.5- to 3-fold higher than in control subjects). In the patient groups, plasma S100A12 is strongly associated with plasma markers of both LDL oxidation and inflammation, and, additionally, with ultrasonically measured carotid atherosclerosis. It is suggested that oxidation processes mediated by copper-bound S100A12 are involved in accelerated atherogenesis in several proinflammatory states.
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S100P regulation and function in cancer

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S100P is an EF-hand calcium-binding protein that was originally identified in placenta and subsequently linked to cancer. It is a member of S100 family of proteins functioning as extracellular and/or intracellular regulators of diverse cellular processes and contributing to various human pathologies. S100P expression was detected in a range of human tumor cell lines and tissues, particularly those derived

from breast, prostate, pancreas and colon, where it was connected with malignant phenotype, hormone independence and resistance to chemotherapy. In line with these observations, forced overexpression of S100P was shown to promote tumorigenesis in prostate, breast and cervical cancer models. Functional studies of S100P indicate that it operates either via intracellular interaction with ezrin, leading to increased cell migration, or via extracellular signaling through RAGE receptor, resulting in increased proliferation and survival. Molecular mechanisms regulating expression of S100P in cancer cells are just starting to emerge. Besides earlier described DNA methylation, recent studies revealed involvement of bone morphogenic protein and non-steroidal anti-inflammatory drugs in control of S100P expression during tumor progression. We performed functional analysis of S100P promoter and identified SMAD, STAT/CREB and SP/KLF binding sites as key regulatory elements participating in transcriptional activation of S100P gene in cancer cells. Moreover, our latest data reveal that expression of S100P is up-regulated by activation of glucocorticoid receptor suggesting that S100P could play a role in therapy resistance mediated by glucocorticoids in solid tumors.

Coexpression and nuclear colocalization of metastasis-promoting protein S100A4 and p53 without mutual regulation in colorectal carcinoma

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Nuclear localization of the metastasis associated protein S100A4 has been shown to correlate with advanced disease stage in primary colorectal carcinomas (CRC), but nuclear function and its relevance for the metastatic capacity of tumor cells is still unclear. Among several nuclear interacting protein partners suggested for S100A4, the tumor suppressor protein p53 has attracted particular interest, and previous studies suggest direct and indirect modes of interaction between the two proteins. The present study was undertaken to assess coexpression and potential interaction in CRC. *TP53* mutational status and S100A4 expression were investigated in a selected series of primary CRC specimens ($n = 40$) and cell lines ($n = 17$) using DNA sequencing, western blot, and double immunostaining. Additionally, S100A4 and p53 were experimentally up and down regulated in vitro to assess reciprocal effects. For the first time, S100A4 and p53 coexpression was demonstrated in individual CRC cells, with nuclear colocalization as a particularly interesting feature. In contrast to previous studies, no correlation was observed between *TP53* mutational status and S100A4 expression, and no evidence was obtained to support reciprocal regulation between the two molecules in the HCT116 isogenic cell line model. In conclusion, S100A4 and p53 were shown to be colocalized in individual nuclei of CRC cells, and it might be speculated whether the proteins interact in this subcellular compartment.

New insights on the role of zinc-calcium interplay in structure and function of human S100A12

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Human S100A12 has a role in inflammation and host parasite responses, and is linked to major diseases such as diabetes, cystic fibrosis and atherosclerosis. Like several other S100 proteins, S100A12 binds zinc in addition to calcium. Zinc binding to S100A12 enhances the calcium affinity by a factor of 1500. Previous studies on the S100A12 interactions with one of its receptors RAGE (the receptor for the advanced glycation end products) show that calcium and zinc dependent oligomerization is essential for target recognition by S100A12. Previously we reported the structures of human S100A12 in both low (dimeric) and high (hexameric) calcium forms, and in addition that of a complex with copper and calcium. Here we present the X-ray structures of S100A12 in the absence of metals (apo form) and in complex with zinc in the absence of calcium. The latter structure is the first of a 'zinc-only' S100 protein complex. The structure explains the influence of zinc on the calcium affinity of S100A12 and allows a model to be proposed for metal-dependent protein oligomerization. We also reveal through an *in vitro* binding assay an important role for both zinc and calcium in S100A12's interaction with paramyosin, its target from the tropical parasites *Onchocerca volvulus* and *Brugia malayi*.

Steps towards a systems biology of the neuron

Principles of computational modeling of neuromolecular mechanisms in mental disorders

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Some psychiatric syndromes can be interpreted as a hyper- or hypo- or dysfunction of mental functions. For instance, in schizophrenia working memory (WM) is instable. Instability implies distractibility, meinaing that low filtering and suppression functions can be performed by the network. Additionally it is known by animal experiments and drug abuse that dopamine (DA) can modulate the WM function. As schizophrenia is related to a hyperfunction of DA transmission, the weak WM function can be explained by high DA. In order to study this concept, computer based simualtions can help to understand the chemical mechanisms of pathological WM. In addition, the cellular and subcellular molecular mechanisms are already studied regarding schizophrenia.

The presentation will demonstrate the research program of systems biology of mental disorders.

Considerations in gene expression profiling: experiment and analysis

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The talk discusses the potential role of quantitative expression profiling in the quest for an improved understanding of complex systems. Current conceptual and statistical challenges in the design and analysis of experiments for the study of gene expression profiles are presented. Both the impact of platform characteristics and questions of high-level inference will be discussed using selected examples from biomedical research.

Molecular systems behind large datasets obtained from microarrays: help form computational approaches

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Typically, large datasets are obtained from microarray experiments. Tables of hundreds of differentially regulated genes may be put in alphabetical order or clustered into functional groups. Both strategies do not provide any clues as to what specific molecular interactions are compromised in the conditions of interest. Disturbances occurring in any disease principally affect the functioning of molecular networks. Therefore, those differentially regulated genes have to be viewed as components of molecular networks in three-dimensional space. This way of thinking is no problem, when only a few genes surface and their functions are well known. Larger numbers of genes make that task prohibitively difficult and confusing, and can only be dealt with by computational means. Some examples of how to process microarray data to eventually gain more detailed insights into disturbances of molecular biological networks will be presented.

Structure and function of excitatory amino acid receptors

Voltage clamp fluoremetry and $GABA_A$ receptors

Weiss David

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Structure-function studies of the Cys Loop family of ionotropic neurotransmitter receptors (GABA, nACh, 5-HT_3, and glycine receptors) have resulted in a six-loop (A–F) model of the agonist binding site. Key amino acids have been identified in these loops that associate with, and stabilize, bound ligand. The next step is to identify the structural rearrangements that couple agonist binding to channel opening. To address this issue, we have been using a technique known as Site-Specific Fluorescence Spectroscopy. In this approach, environmentally-sensitive fluorophores are attached to defined locations on the GABAa receptor. Structural changes around that fluorophore, if they occur, are observed as changes in fluorescence intensity. These fluorescence measurements are carried out in concert with two-electrode voltage clamp in order to assess the level of receptor activation. Using this general approach, we have identified rearrangements in the agonist binding site and are gaining insight into how these structural arrangements ultimately lead to the open (ion conducting) state of the GABAa receptor.

Ion-dependent gating of kainate receptors

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In the vertebrate brain, ligand-gated ion channels are an important class of signaling protein designed to respond to a specific chemical neurotransmitter such as acetylcholine, L-glutamate (L-Glu), glycine, or GABA. Although neurotransmitter substances are numerous, all ligand-gated ion channels are thought to undergo conformations into the activated state by harnessing the energy from neurotransmitter binding. In this talk, I will review recent findings from my lab and others showing that kainate (KA)-selective ionotropic glutamate receptors (iGluRs) require not only the neurotransmitter, L-Glu but also external sodium and chloride ions for activation. Interestingly, AMPA-type iGluRs function normally in the absence of external ions, revealing that even closely related iGluR subfamilies operate by distinct gating mechanisms. This behavior is interchangeable via a single amino acid residue that operates as a molecular switch to confer AMPA receptor behavior onto KARs. Regulation is achieved via functionally-coupled cation and anion binding pockets that constitute a novel gating feature that singles out KARs from all other ligand-gated ion channels. It is still unclear how external ions evolved as co-activators of KARs. Interestingly however, genomes of *C. elegans* and *C. briggsae* contain primitive iGluR-like sequences that are more akin to AMPA receptors. It is therefore tempting to speculate that the gating mechanism of KARs evolved from an ancestral iGluR protein that behaved more like AMPARs. I will speculate on how tracing the evolutionary origin of iGluR gating may provide insight into the possible physiological role that external ions play in regulating KAR function in the vertebrate CNS.

AMPA Receptors and TARPs

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Stargazin and related TARPs (*t*ransmembrane *A*MPA receptor *r*egulatory *p*roteins) are auxiliary subunits of neuronal AMPA receptors. In addition to their role in receptor surface expression and synaptic localization, TARPs also have significant effects on AMPA receptor gating. TARPs slow both deactivation and desensitization by increasing the rate constant for channel opening. TARPs also increase kainate efficacy and speed recovery from desensitization. TARP modulation of receptor kinetics is isoform-specific, being greatest for the TARP isoforms γ-4 and γ-8. Rescuing synaptic transmission in cerebellar granule cells from stargazer mice with different TARP isoforms results in isoform-specific differences in the decay of miniature EPSCs. The slowing of deactivation and desensitization is mediated by isoform-specific sequence elements within the first extracellular loop of TARPs, whereas other TARP domains appear to be involved in modulation of recovery. Comparison of stargazin modulation of receptor kinetics in co-

expression experiments with the corresponding results obtained with a tandem construct in which the C terminus of AMPA receptors is directly fused to the N terminus of stargazin indicate that glutamate-induced desensitization causes the rapid and reversible loss of stargazin modulation of gating. The glutamate-induced loss of stargazin-receptor interactions affects the response of neurons to pairs of stimuli applied at short intervals and may be a mechanism to minimize excitotoxicity when ambient levels of glutamate are elevated.

How clamshell-like domains control NMDA receptor activity?

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NMDA receptors (NMDARs) are glutamate-gated ion channels that play key roles in synaptic physiology and various brain disorders. NMDARs exist as multiple subtypes with distinct pharmacological and biophysical properties that are determined by the type of NR2 subunit (NR2A-D) incorporated in the heteromeric NR1/NR2 complex. Among the most striking features that distinguish NMDAR subtypes is their maximal channel open probability (Po). While NR2A-containing receptors have a relatively high Po (0.5), NR2B-containing receptors are significantly less active (0.1), and NR2C- and NR2D-containing receptors open even more rarely (0.01). By conferring unique charge transfer capacities on each receptor subtype, these differences in Po are thought to be crucial in determining the involvement of NMDAR subtypes in specific neuronal processes. We aimed at identifying the molecular mechanisms accounting for these profound differences in NMDAR activity. Surprisingly, we find that the subunit-specific gating of NMDARs does not originate from the basic gating core module—the agonist-binding domains (ABDs) plus the pore region—but rather from the distal N-terminal region. This region, which encompasses the NR2 extracellular clamshell-like N-terminal domain (NTD) and the short NTD-ABD linker, was previously shown to bind allosteric modulators. We now show that, in the absence of any allosteric ligand, the NR2-NTD is capable of spontaneous oscillations between a closed- and an open-cleft conformation, and that differences in this conformational equilibrium within the NR2 family largely determine the subtype specificity of NMDAR Po. In conclusion, our data reveal how the NR2-NTDs create functional diversity in NMDARs.

When and why amino acids?

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That some amino acids operate as synaptic transmitters in the vertebrate CNS was a great surprise when first suggested 50 years ago. It should not have been. Both glutamate and GABA were later found to be intercellular messengers, throughout the animal kingdom (and indeed many plants, and even protozoa). Glutamate is a very common amino acid—a major constituent of proteins—and produced by several metabolic pathways. Being highly flexible and strongly (negatively) charged, it can bind to different receptor sites in a variety of configurations: these are optimal properties for a protean messenger, able to convey signals to different membrane receptors. So why, in vertebrates is it almost exclusively an excitatory transmitter? Would it not be simpler to have a single synaptic transmitter, capable of binding to inhibitory and excitatory receptors? This solution has not been generally adopted. Excitation is generated by glutamate-induced Na^+ and Ca^{2+} influx; and inhibition by GABA- or glycine-induced Cl- influx. There must have been some advantage in having separate agents, released by different cells: probably greater flexibility in circuit design and operation, especially of the inhibitory systems, which are notoriously subject to modulation by endogenous factors or by drugs. Through regulated changes in internal [Cl], the activation of Cl- channels may paradoxically be excitatory: during early development: excitation by precocious GABAergic transmission is an important stimulus for neuronal and synaptic growth and functional maturation. Such Cl- efflux-mediated depolarization is retained at certain sites where GABA or glycine modulates transmitter release from glutamatergic terminals.

Sulfur- and seleno-containing amino acids

Cysteine dioxygenase: from molecule to mouse

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Cysteine catabolism in mammals may occur by either cysteinesulfinate-independent or cysteinesulfinate-dependent pathways. The cysteinesulfinate-dependent pathway is initiated by the oxidation of cysteine to cysteinesulfinate by cysteine dioxygenase (CDO), an enzyme that adds molecular oxygen to the sulfur of cysteine, converting the thiol to the sulfinic acid; it is very low in liver of animals fed low protein diets but increases markedly within 24 h of an increase in either the protein or sulfur amino acid content of the diet. CDO is one of the most highly regulated metabolic enzymes responding to diet that is known. It undergoes up to 45-fold changes in concentration, via cysteine-responsive regulation of its ubiquitination and degradation by the 26S proteasome, and up to 10-fold changes in catalytic efficiency, as a consequence of turnover-associated thioether bond formation between active-site cysteine and tyrosine residues. This provides a remarkable responsiveness of the cell to changes in sulfur amino acid availability: the ability to decrease CDO activity and conserve cysteine when cysteine is scarce and to rapidly increase CDO activity and catabolize cysteine to prevent cytotoxicity when cysteine supply is abundant. CDO in both liver and adipose tissues responds to changes in dietary intakes of protein and/or sulfur amino acids over a range that encompasses the requirement level, suggesting that cysteine homeostasis is very important to the living organism. CDO-null mice had markedly reduced liver and plasma taurine levels (<5% of control), demonstrating the dominant role played by the CDO-mediated pathway in taurine production and taurine status, even in mice receiving taurine in the dam's milk or in the nonpurified diet.

Hydrogen sulfide as a signal molecule as well as a neuroprotectant in the brain

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Hydrogen sulfide (H_2S) is a synaptic modulator as well as a neuroprotectant in the brain. Cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3MST) produce H_2S from

cysteine in the brain. CBS is mainly expressed in astrocytes, while 3MST in neurons. H_2S selectively enhances the NMDA receptor-mediated responses and facilitates the induction of hippocampal long-term potentiation (LTP), a synaptic model of memory and learning. In astrocytes, H_2S increases the intracellular concentrations of Ca^{2+} that propagate into neighboring astrocytes as Ca^{2+} waves. Neuronal activity evokes glial Ca^{2+} waves, and glial Ca^{2+} waves drive neuronal activity. These observations show that H_2S can act as a signal molecule in the brain. We recently showed that H_2S protects neurons from oxidative stress by increasing the levels of glutathione (GSH), a major cellular antioxidant. H_2S reduces cystine into cysteine in the extracellular space, and enhances the transport of cysteine to increase GSH production in addition to the direct transport of cystine. The efficiency of GSH production enhanced by H_2S is even greater by four-fold under oxidative stress by glutamate. H_2S also causes the distribution of GSH to mitochondria more efficiently than β-me. Cells expressing enzymes involved in H_2S production are significantly resistant to oxidative stress. The present study shows that H_2S protects cells from oxidative stress and that cystine produced by reducing cystine by H_2S is efficiently transported and used to produce GSH. It provides a new mechanism of neuroprotection from oxidative stress by H_2S.

A novel thioredoxin-dependent redox-sensing switch in mercaptopyruvate sulfurtransferase

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Rat 3-mercaptopyruvate sulfurtransferase (MST) possesses two thioredoxin-dependent redox-sensing switches for the regulation of the enzymatic activity. These switches are connected with redox change of each specific cysteine residue. MST contains three exposed cysteines; a catalytic site cysteine, Cys^{247} in the active site, and Cys^{154} and Cys^{263} on the surface of MST. Cys^{247} is a target of oxidants and a stoichiometric concentration of hydrogen peroxide inhibited MST. The activity was completely restored by dithiothreitol or thioredoxin with a reducing system containing thioredoxin reductase and NADPH, but glutathione did not restore the activity. Further, a MALDI-TOF mass spectrometric analysis revealed that mild oxidation of MST resulted in formation of a sulfenate (SO^-) at Cys^{247}, which exhibited a lower redox potential than that of glutathione. It is concluded that a low redox potential cysteine-sulfenate is reversibly formed at a catalytic site cysteine so as to inhibit MST, and thioredoxin-dependent reduction of the sulfenate restores the MST activity. Recently we found that MST had monomer–dimer equilibrium and that MST was oxidized to form an intersubunit disulfide bond between Cys^{154} and Cys^{154}, Cys^{154} and Cys^{263}, and Cys^{263} and Cys^{263} so as to decrease MST activity, and thioredoxin-specific conversion of a dimer to a monomer increased MST activity. It is concluded that an intersubunit disulfide bond in a dimeric MST also serves as a thioredoxin-dependent redox-sensing switch for the regulation of the enzymatic activity of MST.

Reduced sulfur in the plant cell: enzymatic formation and functional roles

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Sulfur can be found in several oxidation states in the cell, either in the free form or as part of organic molecules. This paper describes exemplarily the diverse reactions and putative functions of members of one protein family involved in the transfer of reduced sulfur. The rhodanese/sulfurtransferase (Str) superfamily (Pfam Acc. No. PF00581) is widely distributed in archaea, eubacteria, and eukaryote. Members of this family catalyze the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. To identify putative in vivo substrates a number of compounds have been tested in in vitro assays using several purified recombinant Str proteins from *Arabidopsis thaliana* in different kinds of enzymatic analyses. Conformational analysis done indicate the catalysis of larger molecules, such as proteins, as substrates for some of the Str analyzed. Biochemical data and bimolecular fluorescence complementation demonstrate in vitro and in vivo interaction of specific Str with specific thioredoxins in a compartment-dependent way. These findings and the fact that the expression of various Str is induced by different stress factors let us hypothesize that Str may be involved in the maintenance of the cellular redox homeostasis.

Searching for a functional role of the *Azotobacter vinelandii* sulfurtransferase RhdA: from sulfur delivery protein to "antioxidant" protein

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The ubiquitous rhodanese-domain proteins in vitro catalyse the transfer of a sulfur atom from a suitable sulfur donor (thiosulfate for rhodaneses, 3-mercaptopyruvate for 3-mercaptopyruvate sulfurtransferases) to cyanide, with concomitant formation of thiocyanate. The wide distribution of these proteins (PFAM acc. no.: PF00581), along with the presence of paralogs in the majority of organisms, corroborate the hypothesis that they evolved distinct physiological functions. In *Azotobacter vinelandii* the tandem-domain rhodanese RhdA contains an active-site motif (HCQTHHR) that supports the stabilization of the persulfurated form (RhdA-SSH). The *A. vinelandii* cysteine desulfurases, IscS and NifS, can in vitro mediate the production of RhdA-SSH, in the presence of free L-cysteine as substrate, thus making interesting investigations on the role of the sulfane sulfur bound to RhdA-SSH in driving its in vivo functions. Moreover, the picture that has emerged from the phenotypic characterization of an *A. vinelandii* RhdA null mutant strain indicated that RhdA might trigger protection to oxidative events since the lack of RhdA led to an increased amount of reactive oxygen species (ROS), and growth impairment in the presence of a chemical oxidant (phenazine methosulfate).

Sulfurtransferase and apoptosis induction by natural ally sulfane sulfur compounds: effects on the intracellular detoxification and redox systems

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Thiosulfate-sulfurtransferase (TST) is an enzyme widely distributed in all phyla, involved in the biogenesis of iron-sulfur cluster, in the

cyanide detoxification system and related to the regulation of sulfane sulfur metabolism. However, its biological function is not completely understood and recently, the low activity and/or expression of rhodanese have been also related to the onset of diseases and tumours. The induction of programmed cell death by natural sulfane sulfur compounds (OSCs) opens significant questions concerning the role in the cancerogenesis process of the enzymes, such as TST, involved in their metabolism. Garlic-derived OSCs such as diallyl disulfide, diallyl trisulfide, allicin and more recently the sodium 2-propenyl thiosulfate (2-PTS) have been shown to suppress the proliferation of tumor cells through the apoptosis induction. The biochemical mechanisms underlying the antitumorigenic and antiproliferative effects of these OSCs are not yet fully understood, although it seems likely that the rate of clearance of allyl sulfur groups from cells is a determinant of the overall response. Not all cells are equally susceptible to the deleterious effects of the garlic sulfur compounds and, in particular, non-neoplastic cells tend to be less susceptible probably due to a different expression or activity of the enzymes involved in their metabolism, such as TST. We investigated the effects of 2-PTS on the detoxification and endogenous antioxidant systems in HuT-78 tumor cells and through in vitro interaction studies. Our results promote an involvement of the essential rhodanese-thioredoxin-thioredoxin reductase system in the antiproliferative effects of organo-sulfane sulfur compounds in the tumor cells and, further, suggest a relevant role of the GSH in the anti-proliferating effect of the 2-PTS.

Cysteine *S*-conjugate *β*-lyases

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Many exogenous and some endogenous electrophiles are metabolized/detoxified to mercapturates: electrophile → glutathione *S*-conjugate → cysteinylglycine *S*-conjugate → cysteine *S*-conjugate ⇄ *N*-acetyl cysteine *S*-conjugate (mercapturate) → excretion. However, if the electrophile contains an electron-withdrawing group, the cysteine *S*-conjugate intermediate may undergo a *β*-lyase-catalyzed *β*-elimination reaction: cysteine *S*-conjugate + H_2O → pyruvate + NH_4^+ + mercaptan (RSH). Several pyridoxal 5'-phosphate (PLP)-dependent enzymes, including glutamine transaminase K (GTK), catalyze cysteine *S*-conjugate *β*-lyase reactions. In mammals, the resultant mercaptan may be detoxified by *S*-methylation (thiomethyl shunt), *S*-oxidation or *S*-glucuronidation, and excreted. However, if the mercaptan is highly reactive, the mercapturate/cysteine *S*-conjugate *β*-lyase pathway may result in toxification. Halogenated alkenes and possibly the anti-cancer drug, cisplatin, may be bioactivated in this manner. The anti-cancer drug busulfan is metabolized in part through the *β*-lyase pathway, but the eliminated fragment is a thioether rather than a mercaptan. Selenocysteine *Se*-conjugates are excellent aminotransferase and *β*-lyase substrates of GTK. The propensity of PLP-enzymes to catalyze *β*-elimination reactions might be useful in the design of cysteine *S*- and selenocysteine *Se*-conjugate prodrugs. Several disulfide-containing cysteine *S*-conjugates in allium foods are substrates of mammalian cysteine *S*-conjugate *β*-lyases. The eliminated products are persulfides (RSSH) that exhibit excellent antioxidant potential. Finally, cysteine *S*-conjugate *β*-lyase reactions give rise to odorants, flavorings and fragrances in such disparate sources as perspiration, wines and plants. In conclusion, the mercapturate/cysteine *S*-conjugate *β*-lyase pathway occasionally may be detrimental by generating toxicants, but its ubiquity in nature and diversity of potential substrates can provide biologically useful thiols, persulfides and selenols.

Products of organoselenium (*Se*)-conjugates obtained by action of *β*- and *γ*-lyases, L-amino acid oxidase and aminotransferase(s) are chemopreventive

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Chemopreventive studies on selenoamino acids have focused on their incorporation into antioxidative selenoproteins. Recent studies show that non-protein selenomethionine and the 21st-coded amino acid, selenocysteine, also have anti-cancer properties. The biochemical mechanisms that contribute to their chemoprotective effects involve transformations catalyzed by *β*- and *γ*-lyases, L-amino acid oxidase, and/or aminotransferases. Some enzymes containing pyridoxal 5′-phosphate (PLP) catalyze *β*- or *γ*-lyase reactions with methylselenocysteine (MSC) and selenomethionine (SM), respectively, resulting in generation of an α-keto acid, ammonium and methylselenol. Chemopreventive activity of methylselenol may act through the oxidation of thiol moieties on redox-responsive signal proteins and transcription factors, thereby maintaining a non-proliferative intracellular environment. Several aminotransferases catalyze *β*-elimination reactions with *Se*-conjugates as a result of the strong electron-withdrawing properties of selenium, but competing transamination can ensue providing a keto acid co-substrate is present or PLP coenzyme is replenished. Human glutamine transaminase K catalyzes transamination and *β*-lyase reactions with MSC but not with SM. After transamination of MSC, the resultant methylselenolpyruvate (MSP) increases histone-H3 acetylation in human prostate and colon cancer cells. MSP and the α-keto acid product of SM, α-keto-*γ*-methylselenobutyrate (KMSB) resemble butyrate, an inhibitor of histone deacetylase (HDAC). L-Amino acid oxidase converts MSC and SM to MSP and KMSB, respectively, which inhibit HDAC activity in a dose-dependent manner. Thus, the α-keto acid metabolites of MSC and SM, along with methylselenol derived from *β*- and *γ*-lyase reactions, may be potential direct-acting metabolites of organoselenium that lead to de-repression of silenced tumor suppressor proteins and/or responses to signal factors.

New synthetic strategies towards selenocysteine and selenomethionine derivatives

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Selenium-containing amino acids have attracted increasing interest from view points of the importance as a active center of several selenoenzymes, the biological synthesis, the metabolism, and the use for structure determination of proteins. A number of efficient synthetic methods have already been developed for L-selenocysteine (SeCys) and L-selenomethionine (SeMet) derivatives. In most of such methods, however, the starting material was rather limited to L-serine (Ser) or L-homoserine and the transformation was usually achieved in a step-wise manner through the activation into the tosylate, halide, beta- or gamma-lactone, and oxazoline intermediates. In this paper, we present our recent progresses in the development of new strategies for the synthesis of SeCys and SeMet derivatives.

The use of L-cystine (Cys2), instead of Ser, as a starting material was first investigated. After protection of the amino and carboxy groups, the Cys2 was treated with iodine and triphenylphosphine in the presence of a base to yield the corresponding beta-iodoalanine derivative. The iodide was subsequently reacted with sodium hydrogen selenide (NaHSe) to afford the SeCys derivative in ca. 70% yield. The similar transformation was applied for derivatization from homocysteine to homeselenocysteine and SeMet derivatives. The S to Se modification (i.e., the chemical atomic mutation) would be a useful approach to the peptide, which involves a selenoamino acid residue at a specific position, from a naturally occurring Cys-containing peptide. One-step conversion of Ser to SeCys derivatives as well as antioxidant catalytic activities of the synthesized SeCys and SeMet derivatives was also investigated.

Novel methionine-selective transporters from the neurotransmitter sodium symporter family

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Nutrient amino acid transporters (NATs) comprise a recently identified subfamily of the Neurotransmitter sodium symporter family (NSS or SLC6). It includes a large population of orphan transporters that form lineage-specific basal clusters of the SLC6 family tree. We report the cloning, functional expression, and in situ hybridization of a novel insect NAT from the yellow fever vector mosquito, Aedes aegypti, AeNAT5 (NCBI accession, ABZ81822). In contrast to the previously characterized Broad spectra transporter AeAAT1 and the tryptophan- and phenylalanine-substrate selective transporters from another culicid mosquito, Anopheles gambiae (AgNAT6 and AgNAT8), AeNAT5 selectively absorbs L-methionine and with a 20-fold reduction in transport efficiency L-cysteine. AeNAT5 transcript mimics the general pattern of spatial expression of NATs in the insect gut. However, some substantial differences in the expression of the AeNAT5 versus AeAAT1 were identified in the anterior and posterior domains of the larval alimentary canal. These findings support our earlier hypothesis that the NAT subfamily evolved and act synergistically as the principal molecular mechanisms for essential amino acid absorption and redistribution. The narrow substrate spectra AeNAT5, AgNAT6 and AgNAT8 represent a specific expansion of insect NATs which amplify the selective acquisition of the most underrepresented essential amino acids. Essential NATs are important for animal health and brain function, and their strong lineage-specificity compared to conserved neurotransmitter transporters suggests that NATs could be more rational targets for the management of medically and economically important invertebrates.

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Can the level of sulfane sulfur be a marker of neoplastic cell transformation?

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Cystathionine gamma-lyase (CST) is the rate-limiting enzyme for the synthesis of cysteine from methionine and the availability of cysteine is a critical factor in glutathione synthesis. CST and 3-mercaptopyruvate sulfurtransferase (MPST) catalyse reactions of the formation of sulfane sulfur-containing compounds from L-cysteine. Tissue concentration of glutathione and sulfane sulfur is relevant to the malignant cell proliferation. Our investigations have demonstrated a higher concentration of GSH and cysteine, and a decreased activity of CST and MPST in human brain gliomas as compared to non-involved tissues (various regions of human brain). The level of sulfane sulfur in brain gliomas seems to be dependent on the grade of malignancy (according to the WHO classification). The application of the micro-XANES technique, as opposed to the biochemical bulk analysis, has allowed for showing that a high accumulation of sulfur as the sulfide (S^{2-}) form occurred just inside the cancer cell. In the astrocytoma U373 cells, as compared to astrocytes, the diminished activity of MPST is accompanied by a lower level of sulfane sulfur. *N*-acetyl-L-cysteine (NAC), a precursor of cysteine, increases the level of sulfane sulfur in the astrocytoma U373 and neuroblastoma SH-SY5Y cells, thus inhibiting their proliferation. The interrelationship between cell proliferation and sulfane sulfur level has been confirmed for the prostatic cancer cells PC-3 cultured in a medium containing diallyl disulfide (DADS, an organosulfur compound of garlic). The inhibition of PC3 cells proliferation has been accompanied by an increased expression of CST, while the proliferation of SH-SY5Y cells by an increased expression of MPST. The cellular sulfane sulfur levels strictly depend on the activity of sulfurtransferases; loss of these enzymatic activities may result in sulfane sulfur accumulation in brain gliomas. It seems that the modulation of MPST and/or CST expression may have a therapeutic importance.

Determination of homocysteine redox status in urine by liquid chromatography

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Thiol redox status, defined as reduced-to-oxidized ratio, of urinary homocysteine (Hcy) has been measured. Both forms of Hcy were determined by high performance liquid chromatography with fluorescence detection. The analyte was on-column derivatized with fluorescence labeling reagent, *o*-phthaldialdehyde, separated chromatographically and detected at excitation and emission wavelengths 370 and 480 nm, respectively. Oxidized form of Hcy was converted to its thiol counterpart by reductive cleavage with tris-(2-carboxyethyl)phosphine hydrochloride prior to derivatization step.

The calibration graphs, obtained with the use of normal urine spiked with growing amounts of homocysteine and homocystine, were linear over the concentration ranges covering most experimental and clinical cases (0.05–8 nmol/ml urine for reduced homocysteine and 0.25–16 nmol/ml urine for total homocysteine). The oxidized homocysteine was calculated by subtraction of reduced thiol from the content of total thiol. Redox status of homocysteine was determined in urine of 32 healthy volunteers distributed by sex. Excretion of Hcy, normalized against creatinine, showed a wide range of variation, however mean values were not significantly different ($P < 0.05$) between sex groups. Our investigations show positive correlation between reduced and total homocysteine in first morning urine ($r = 0.829$).

Total cysteine, glutathione and homocysteine profiles of fruit and vegetable juices using capillary electrophoresis with pH-mediated sample stacking

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Biological thiols are important antioxidants, and recent studies showed that their contents vary depending on the groups of foodstuffs. Therefore, we investigated the levels of some biological thiols in various vegetables and fruits using a sensitive high-performance capillary electrophoresis (HPCE) technique. The method is based on reduction of disulfide bonds, derivatization of thiols with 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT), followed by electrophoretic separation with pH-mediated staking step and UV-absorbance detection and quantitation. When using capillary electrophoresis for the analysis of biological samples, it is often necessary to employ techniques to overcome peak-broadening that results from having a high-conductivity sample matrix. To improve the concentration detection limits and separation efficiency of the cationic CMQT derivatives in HPCE, pH-mediated acid stacking was performed to electrofocus the analytes, improving separation sensitivity. Biological thiols measured in some vegetables and fruits include cysteine, glutathione and homocysteine. Our results show that various vegetables and fruits differ significantly in their content of thiols. We confirmed that the main nonprotein thiols in plants are cysteine and glutathione. The method is linear in wide range of concentrations with a regression coefficient better than 0.99. The detection limit for glutathione, cysteine and homocysteine were 2.5 μmol/L. The method was successfully applied to analysis of fruit and vegetable juices for total cysteine, glutathione and homocysteine.

The activity of the hydrogen sulfide generating enzymes: cystathionine β-synthase and γ-cystathionase—in mouse tissue homogenates

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Hydrogen sulfide (H_2S), an important gasotransmitter with a vasorelaxant property, is generated endogenously in vascular smooth muscle cells and nervous system. It can be produced from L-cysteine by one or two pyridoxal-5′-phosphate-dependent enzymes present in mammalian tissues: cystathionine β-synthase (CBS) and γ-cystathionase (CST). CBS synthesizes cystathionine, while CST splits cystathionine to cysteine, α-ketobutyrate and ammonium ions. We investigated the activity of these two enzymes in mouse liver, kidney and brain homogenates in the presence of homoserine as the CBS substrate and in the presence of DL-propargylglycine (PAG) as the CST inhibitor. The levels of cystathionine, cysteine, α-ketobutyrate and glutathione were analyzed in incubation mixtures, after 15 min incubation with 8 mM homoserine, using the RP-HPLC method. The highest control level of cystathinine was detected in the brain (70 pmole/mg protein) and it increased twofold in the investigated sample. The difference in the cystathionine level between the homogenates with and without PAG (0.1 mM) can be used to estimate the activity of CBS in tissue homogenates. The mouse brain homogenate showed CBS activity that was about ten times higher (4.7 pmole/mg protein min) in comparison to the liver. The combined activity of the CBS and CST measured by the difference between cysteine and/or α-ketobutyrate levels in the control and investigated samples was also the highest in the brain homogenates as compared to the liver and kidney. This suggests higher brain potency in H_2S generation. The highest increase in the level of glutathione was found in the liver homogenates, what points both to the role of the CBS/CST tandem in cysteine delivering and to the high activity of enzymes participating in glutathione synthesis.

The effect of diallyl trisulfide on neuroblastoma SH-SY5Y cells proliferation

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Diallyl trisulfide (DATS), a constituent of processed garlic, inhibits proliferation of human cancer cells. We intended to elevate the level of sulfane sulfur in neuroblastoma SH-SY5Y cells by delivering DATS, sulfane sulfur donor. The effects of various concentrations of DATS on cell's proliferation, glutathione (GSH) content, and sulfurtransferases activity were investigated in human neuroblastoma SH-SY5Y cells. Cells were treated with 0 (control), 1.0, 2.0 or 3.0 mM DATS for 24 or 48 h. After 24 and 48 h of treatment with 2.0 mM DATS the inhibition of cells proliferation was observed. The effect was accompanied by an increased expression of γ-cystathionase and 3-mercaptopyruvate sulfurtransferase (MPST), two sulfane sulfur-synthesizing enzymes. The elevated activity of MPST and the increased level of sulfane sulfur were also determined. Our results suggest that a correlation exists between the proliferation of SH-SY5Y cells and the level of sulfane sulfur and the expression / activity of MPST and γ-cystathionase.

The involvement of calpain in homocysteine-induced apoptosis in SH-SY5Y cells

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Previous studies have reported the endoplasmic reticulum (ER) stress caused by homocysteine, proposing the underlying mechanism by which it could induce neurodegeneration. The calpains are a superfamily of Ca^{2+}-dependent proteases that may play an important role in ER stress-mediated apoptosis in neuronal cells. We investigated whether calpain is involved in homocysteine-mediated apoptosis in SH-SY5Y human neuroblastoma cells. Homocysteine increased intracellular Ca^{2+} level and calpain activity in a concentration-dependent manner. Expression of Bax, cytochrome *c* release from the mitochondria, and activation of caspase-9 increased significantly in cells treated with homocysteine. Consistently, the viability of cells cotreated with homocysteine and a caspase-9 inhibitor increased significantly relative to that of homocysteine-treated cells. Moreover, calpain inhibition significantly increased cell viability and inhibited caspase-9-mediated apoptosis relative to those of homocysteine-treated cells. Taken together, our findings suggest that calpain and the mitochondrial pathway are involved in homocysteine-mediated apoptosis in SH-SY5Y cells.

S-Carbamoylation impairs the radical scavenging activity of cysteine

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Carbamoylation of free amino acids, peptides and proteins is the reaction of urea-derived cyanate ($^{-}$N=C=O)/isocyanic acid (H–N=C=O) with amino, hydroxyl and thiol groups. Carbamoylation can result in altered biological function/activity of the modified molecule due to changed structure and charge.

Most of the investigations dealing with carbamoylation focus on the reaction with the ε-amino groups of proteins (*N*-carbamoylation). Beyond that, the reaction of cyanate at moderate acidic pH (pH 6–7) with the sulfhydryl group (*S*-carbamoylation) is fast. Carbamoylation is observed in vivo in patients suffering from uremia, and uremia has been linked with impaired antioxidant mechanisms. Thio amino acids have been proposed as important in vivo antioxidants due to the radical scavenging ability of the free sulfhydryl (-SH) group. Thus, one may speculate that carbamoylation of the -SH group may impair the antioxidative activity of cysteine (and related thiol compounds). Here we report on the effect of *S*-carbamoylation on the radical scavenging ability of cysteine (CYS), *N*-acetyl-cysteine (NAC) and glutathione (GSH). *S*-carbamoylation of CYS, NAC and GSH resulted in impaired scavenging of free radicals as tested in the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Lipid oxidation in LDL (low density lipoprotein) by the free radical generating compound AAPH (2,2′-azobis-2-amidinopropane hydrochloride) was inhibited by CYS, NAC and GSH. *S*-carbamoylation of the compounds resulted in less inhibitory activity. These results indicate that *S*-carbamoylation of free cysteine may contribute to the impaired antioxidant defense observed in patients suffering from uremia.

Cellular biosynthesis of *S*-adenosylmethionine by Immobilized recombinant *E. coli*

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The problems inherent in microbial fermentation and chemical synthesis of *S*-adenosylmethionine (SAM) led us to develop a whole-cell catalytic method for the synthesis of large scale of SAM (see the synthesis scheme below). In the present study, the full-length SAM synthase gene was cloned from the genome of E.coli K12 and over-expressed in *E. coli* JM109. In order to enhance the expression level of soluble SAM synthase, various fermentation conditions were optimized including temperature, density of IPTG and induction time. Subsequently, the cultured cells were harvested and embedded in sodium alginate, carrageenan gel and gelatin respectively for the synthesis of SAM. The sodium alginate was selected for the better enzyme recovery and mechanical strength. Immobilized cell has been used as efficient catalyst for large scale synthesis of SAM directly from ATP and L-methionine. The synthesis procedures do not require time-waste protein purification and the immobilized cells can be directly used after being simply separated and harvested with filter or centrifuge. The biosynthetic conditions were optimized including pH, temperature, reaction time, catalyst amount, ATP concentration, organic penetrants and inorganic salts concentration. The highest yield of SAM, 13.4 g/L, was obtained when initial ATP concentration was 35 mM at pH 6.5, 35°C. The conversion rate of ATP was over 95%. After five repeated experiments, the activity of SAM synthase with the immobilized cells retained over 90% of their original activity, which indicated that the operational stability for recycling in batch processes was improved. Therefore, immobilized whole-cell catalysis is an efficient, low cost method for producing SAM.

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Cysteine overproduction by engineered *Escherichia coli* strain with a high ethanol yield

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In *Escherichia coli*, intracellular L-cysteine content is strictly controlled and cannot be overproduced in the cell. To overproduce L-cysteine in *E. coli*, we have so far overexpressed feedback inhibition-insensitive altered serine acetyltransferase (SAT), a key enzyme in L-cysteine biosynthesis pathway, lowered level of cysteine desulfhydrase activity which degrade L-cysteine to pyruvate, ammonia, and sulfide, and developed the efficient export system of L-cysteine. However, it is thought that acetic acid, which is one of the by-products in the biosynthesis, is also produced nearly equal to L-cysteine. Acetic acid would result in a negative influence on the cell growth due to decreasing in the pH of the medium. Meanwhile, the most efficient ethanologenic *E. coli* strain was constructed by inverse metabolic engineering based on elementary mode analysis. Strain TCS083, with eight gene knockout mutations (*zwf, frdA, ldhA, sfcA, maeB, ndh, pox, pta*), was found to yield ethanol from glucose closely matched the theoretical predictions with very low acetic acid yields. By introducing the L-cysteine high-producing plasmid into strains wild-type MG1655 and TCS083, the L-cysteine yield per glucose consumption in TCS083 transformant was remarkably higher than that of the wild-type cell. These results suggest that the elimination of organic acid-producing pathway as by-product is important for the improved productivity of L-cysteine.

Synthesis of selenocysteine derivatives and their antioxidant catalytic activities

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Selenocysteine is the 21st amino acid incorporated in proteins by the genetic codon and usually located at the active center of redox selenoenzymes, such as glutathione peroxidase (GPx). We have recently developed the transformation reaction from disulfides to diselenides through iodization and applied the reaction to the synthesis of selenocysteine derivatives. In this paper, improvement of the reaction yield, synthesis of a selenoglutathione derivative by the solid phase peptide synthesis method and evaluation of redox catalytic activities of the synthesized compounds will be reported.

By using L-cystine as a starting material, we synthesized L-selenocysteine derivatives as follows. Amino groups of L-cystine were protected with Fmoc groups, and the carboxy groups were transformed to the methyl ester in 74% total yield. The resulting disulfide was reacted in benzene with triphenylphosphine and iodine in the presence of DMAP to yield the iodide. The subsequent treatment with sodium hydrogen selenide produced a protected L-selenocystine in 72% yield. Ester groups of the obtained diselenide were hydrolyzed under acidic conditions, and the diselenide group was protected with a *p*-methoxybenzyl group by the treatment with potassium borohydride and *p*-methoxybenzyl bromide in THF and water. The total yield of the final selenocysteine derivative was 29%.

We subsequently investigated the synthesis of a selenoglutathione derivative from the obtained selenocysteine derivative. Redox catalytic activities of the synthesized selenocysteine derivatives were also evaluated by using the GPx model reactions between hydroperoxides and thiol substrates.

Electrochemical detection of low molecular mass thiols

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Sulfur exists in multiple stable oxidation states, hence it is a versatile component in biological systems. The most active and reduced form of sulfur in biomolecules is the thiol group (-SH), present in number of biologically active compounds such as the amino acid cysteine, the non-protein forming aminoacid homocysteine and glutathione, which is the most abundant non-protein molecule in mammalian cells. The main aim of this paper is to utilize high performance liquid chromatography with electrochemical detection (HPLC-ED) for determination thiols content in plants tissues of lettuce treated with lead(II) ions (0, 0.5 and 1 mM), respectively. We used two HPLC-ED instruments: HPLC coupled with one channel amperometric detector and HPLC coupled with twelve channel coulometric detector to detect simultaneously twelve thiols (cysteine; reduced, oxidized and *S*-nitroso glutathione; homocysteine; *N*-acetyl cysteine; cystine; phytochelatins: des-Glycine-Phytochelatin, phytochelatin2, phytochelatin3, phytochelatin4 and phytochelatin5). The detection limits of thiols measured by CoulArray detector were about two magnitudes lower in comparison to those measured by Coulochem III detector and were from tens to hundreds pM. Under the optimal conditions (gradient profile for simultaneous thiol separation starting at 100:0 (80 mM TFA:methanol) kept constant for 9 min, then decreasing to 85:15 during 1 min, and kept constant for 8 min, and finally increasing linearly up to 97:3 from 18 to 19 min, mobile phase flow rate of 0.8 ml/min, and column temperature of 40°C) we utilized HPLC-CoulArray detector for analysis of tissues from lettuce plants. **Acknowledgments:** Financial support from GACR 522/07/0692 and INCHEMBIOL MSM0021622412 is greatly acknowledged.

Synthesis and medicinal chemistry

An efficient total synthesis of kaitocephalin, a potent antagonist of iGluRs

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Kaitocephalin, isolated from *Eupenicillium Shearii* PF1191 by Seto and Shin-ya et al. exhibits potent neuro-protecting activity from the excitotoxicity induced by NMDA, AMPA, and KA in rat hippocampal neurons. The structure consists of different type of three amino acids (Ala, Pro, and Ser) connected with carbon-carbon bonds in which the Pro-Ser unit involves three-contiguous stereogenic centers including quaternary carbon center attached to an amino group. Because of its important biological activity and intriguing structure, kaitocephalin has attracted significant attention from chemists as a synthetic target and as a lead of neuroprotecting drugs. Three total syntheses including our work have been reported. However, it still remained an alternative method that enables gram scale preparation of kaitocephalin to examine details of its pharmacological profile and to perform the SAR studies. In this report, we describe an efficient total synthesis of kaitocephalin. The synthesis was performed by (1) diastereoselective aldol condensation of 5-substituted proline ester with an ortho ester protected serinal, (2) inversion of the stereogenic center at C3 of the aldol product, (3) trans-esterification of the ortho ester to the methyl ester, (4) *E*-selective Horner-Emmons olefination using a glycine phosphonate, (5) Rh-catalyzed hydrogenation of the unsaturated ester to the 9*S*-isomer, and (6) one-pot removal of all protecting groups using hard-soft concept to give kaitocephalin. Total number of processes included in 12 steps (6.9% overall yield). Preliminary results for the neuro-protecting and binding activities of kaitocephalin and its analogs will also be described.

Development of new generation of nucleophilic glycine equivalents for general synthesis of alpha-amino acids

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This lecture will review the advances in the synthesis of sterically constrained alpha-amino acids, specifically the preparation of

optically active beta-substituted pyroglutamic acids via the homologation of a new generation of modular Ni(II) complexed glycine equivalents. Following a brief overview of the general methods for the preparation of alpha-amino acids via homologation of achiral glycine equivalents, the majority of this lecture is dedicated to the design, development and application of a new generation of modular nucleophilic glycine equivalents. The key advantage to this new series of glycine equivalents is the ability to alter the physical and chemical properties of these derivatives to fit the requirements of the desired homologation technique. This modular approach has already been exploited in order to fine tune the Ni(II) complexed glycine equivalent for the asymmetric synthesis of a variety of beta-substituted pyroglutamic acids via its Michael addition reaction with various (*S*)-3-(*E*-enoyl)-4-phenyl-1,3-oxazolidin-2-ones. New results on catalytic version of these Michael addition reactions with application of chiral organic bases will highlighted.

Chemical syntheses of radioisotopes and stable isotopes labelled amino acids. An overview of works carried out at "CEA Service des Molécules Marquées" from 1949 to 1989

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The "Commissariat à l'Energie Atomique" (CEA) was created by General de Gaulle shortly after the second world war nightmare, and the author was hired by Dr J. Guéron in September 1949 to start from scratch what became the CEA "Service des Molécules Marquées" (SMM).

Among other subjects of interest to biochemists, the synthesis of labelled amino acids occupied an important place. Examples are mentioned below.

[^{14}C] Amino acids. ^{14}C, issued from neutron irradiation of ^{14}NAl, is commercially available as $^{14}CO_3Ba$ from which "basic" compounds were prepared, e.g. $^{14}CO_2$; K^{14}CN; [$^{14}C_2$] acetylene; [$^{14}C_6$] benzene. The carbonation of organometallics with $^{14}CO_2$ were used as the first step in syntheses of, e.g., [2-^{14}C] amino acids; [4-^{14}CO] δ-amino levulinic acid; [1-^{14}C] γ-amino butyric acid; hydroxy proline; allo-hydroxy proline, etc. Many examples of [^{14}C] labelling using K^{14}CN will be shown on the poster, e.g. [5-^{14}C] glutamic acid; ornithine; arginine, etc. [$^{14}C_2$] acetylene and a Wittig reaction yielded [3,3',4,5-^{14}C] DL-isoleucine. Co-cyclotrimerisation of [$^{14}C_2$] acetylene with diethyl propargyl formamidopropionate and subsequent hydrolysis produced [1,2,3,4-^{14}C] phenyl alanine in a 3-step synthesis with a 30% overall yield.

[^{35}S] Amino acids: cystine; cysteine; methionine.

[^{3}H] Amino acids: L-lysine; L-leucine; L-ornithine; L-arginine; DL-glutamic acid with a high specific radioactivity; meso-diamino pimelic acid.

The SMM also had a prominent position in other syntheses:

- ^{3}H-D PAT, a new ligand in 5HT1A receptors;
- nucleosides (^{3}H and [^{14}C] thymidines);
- fatty acids, e.g. [^{14}C] erucic acid;

Custom syntheses by the SMM of new drugs or candidates of new drugs (metabolism and pharmacokinetic studies) were awarded the Ehrlich Prize/Janssen at the 44th International Meeting on Medicinal Chemistry in July 2008.

The trafficking of amino acid activated Ligand gated ion channels and the control of neuronal activity Dissecting out the role of DISC1 in Schizophrenia through protein-protein interactions at the synapse

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*D*isrupted *i*n *Sc*hizophrenia *1* (DISC1) is an increasingly well-validated psychiatric risk gene which has now been shown to be involved in key signaling pathways with relevance to disease such as wnt and cAMP pathways. In our original studies we generated a network of protein-protein interactions (PPIs) around DISC1, achieved by utilizing iterative yeast-two hybrid screens, combined with detailed pathway and functional analysis. This so-called 'DISC1 interactome' contains many novel PPIs and provides a molecular framework to explore the function of DISC1.

To understand the role of DISC1 at the synapse we have focused on the interaction with Traf-2- and Nck-interacting kinase (TNIK), a member of the Ste20-related kinase family. TNIK has recently been associated with cognitive deficits in schizophrenia. To date little is known of its role in the brain and disease processes. We have shown that TNIK is enriched in the hippocampus and is found in the post-synaptic density of hippocampal neurons. I will discuss the role of a DISC1-TNIK complex at the synapse and suggest that deficits of a TNIK-DISC1 signalosome may contribute to altered synaptic transmission and the pathogenesis of psychiatric disorders.

Tryptophan catabolism and immune regulation

Deciphering the lexicon between fungi and mammalian innate immunity along the kynurenine pathway

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The immune system continuously modulates the balance between responsiveness to pathogens and tolerance to non-harmful antigens. The mechanisms that mediate tolerance are not well understood, but recent findings have implicated tryptophan catabolism through the kynurenine metabolic pathway as one likely mechanism involved. Initially recognized in infection because of its antimicrobial activity, the enzyme indoleamine 2,3-dioxygenase (IDO), by mediating tryptophan catabolism, has a complex role in immune homeostasis

of the mammalian host. The inflammatory–anti-inflammatory state of dendritic cells and neutrophils in response to *Candida* and *Aspergillus*, two major fungal pathogens in humans, is strictly controlled by tryptophan catabolism and mediated by the enzyme IDO through IFN-γ and CTLA-4-dependent mechanisms. IDO blockade greatly exacerbated infections and the associated inflammatory pathology, and swept away resistance to re-infection as a result of deregulated innate and adaptive immune responses caused by the impaired activation and function of suppressor T regulatory cells producing IL-10. According to this model, in chronic granulomatous disease (CGD), a genetic disorder characterized by lack of nicotinamide adenine dinucleotide phosphate oxidase, a host's failure to produce reactive oxygen species—mostly notably, superoxide anion—leads to IDO dysfunction and pathogenic inflammation. Replacement therapy with natural kynurenines reversed the inflammatory phenotype, raising the possibility that this might help to control CGD in humans. The fine control exerted over innate and adaptive antifungal responses by IDO provides novel mechanistic insights into complex events at the host–pathogen interface that may have important therapeutic implications.

HIV-induced tryptophan catabolism: pathogenic mechanism and target for immunotherapy

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The hallmark of human immunodeficiency virus (HIV) infection is the progressive loss of CD4 and CD8 T cell responses, resulting in the inability of the immune system to efficiently respond to opportunistic infections and to HIV itself. Tryptophan (Trp) degradation into kynurenine (Kyn), mediated by indoleamine-2,3-dyoxigenase (IDO) is a powerful physiologic immunosuppressive system. We showed that HIV directly induces IDO expression by plasmacytoid dendritic cells (pDC) in vitro, and that HIV-induced IDO inhibits CD4 and CD8 T cell proliferative responses. The IDO inhibitor 1-methyl-D-tryptophan efficiently prevented IDO-mediated immunosuppression in vitro. We then tested IDO expression in lymphoid and mucosal tissues of rhesus macaques infected with simian immunodeficiency virus (SIV), a well accepted animal model for HIV infection. We found elevated levels of IDO in tissues from animals with high viral load compared to animals with low virus load and uninfected animals. We administered the IDO inhibitor 1-methyl-D-tryptophan (D-1mT) for 13 days to SIV-infected rhesus macaques receiving antiretroviral therapy (ART). D-1mT treatment increased the plasma levels of Trp, without significantly reducing Kyn, suggesting only a partial effect on IDO enzymatic activity. Surprisingly, D-1mT significantly reduced the virus levels in plasma and lymph nodes of ART-treated animals which did not fully respond to ART. In SIV-infected animals which were not receiving ART, D-1mT was ineffective in reducing the plasma viral load. Thus, D-1mT appeared to synergize with ART in inhibiting viral replication. Further studies are required to elucidate the immunologic or virologic mechanism by which D-1mT inhibited SIV replication in vivo.

Regulation of antigen specific immune responses during infection: is there a role for IDO?

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The Tryptophan catabolizing enzyme IDO has turned out to play a great but very divers role in immune regulation. Trp is not only necessary for growth of microorganism, but also essential for the induction antigen-specific T cell responses. Therefore, control of Trp supply by IDO exhibits the contradicting effect to inhibit pathogen growth but at the same time prevents T cells from eradicating the infection by blocking T cell activation or by inducing regulatory T cells. It therefore seems likely that these conflicting events are separated by time and location.

Initially, we had observed that the systemic injection of bacteria or toll-like-receptor-Ligands (TLR-Ligands, CpG-DNA) induced the production of IDO in the spleen, but not in lymph nodes. This IDO activity prevented T cell proliferation and thereby effector function. As control of T cell activity by IDO was intriguing, we wanted to extrapolate these results to the situation of cerebral malaria, a fatal disease, which strongly depends on the action of activated T cells infiltrating the brain. Activation of T cells causing the pathology occurs in the spleen and as expected injection of CpG-DNA could indeed block the onset of cerebral malaria. These results implied that control of IDO activity in the spleen could have a protective effect during malaria, as IDO activity could control T cell function in the spleen, prevent T cell activation ("induction phase") and subsequent damage in the brain.

Indoleamine 2,3-dioxygenase in human hematopoietic stem cell transplantation

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Hematopoietic stem cell transplantation (HSCT) in humans is predictably followed by a severe immunocompromise in the host as caused by the preparative regimen, the infusion of stem cells and the immunosuppressive medication to prevent rejection and graft-versus-host disease. We recently discovered that post-HSCT monocytes suppress post-HSCT T-cell responses. Post-HSCT monocyte suppressor activity was associated with the release of the tryptophan metabolite kynurenine. Indeed, post-HSCT monocytes but not control monocytes were in a constitutively activated state (by increased neopterin release) and were highly susceptible to release high amounts of kynurenines upon activation with even small amounts of interferon-γ (IFN-γ), suggesting augmented IDO activity in the post-HSCT period. In view of the involvement of IDO activity after HSCT and its presumed tolerogenic properties we are now studying whether a targeted IDO induction in human monocyte-derived dendritic cells (mDCs) will eventually result in allo-antigen-specific tolerance. To this end, we found that (1) abundant IDO competence (IDO protein expression and enzymatic

activity) can be induced in human mDCs upon activation with lipopolysaccharide and IFN-γ, (2) IDO competent mDCs can suppress and/or induce regulatory activity in allogeneic T cells and (3) by preliminary findings IDO competent mDCs induce apoptotic decline preferentially of allo-activated T cells. Collectively, these findings suggest that employing IDO competence in human mDCs might be a useful approach to ex vivo tolerize T cells specifically towards allo-antigens and that T cells tolerized via the IDO pathway might be useful for post-HSCT adoptive cell transfer strategies.

Urea cycle and amino acid transport disorders

Newborn mass urine screening for urea cycle and transport amino acid disorders

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The Provincial Mass Urinary Screening Program for inherited metabolic disorders was instigated more than 35 years ago in the Province of Quebec and the Nunavut region as part of a preventive genetic medicine program. It is supported by the Quebec Ministry of Health and Social Services. More than 2,600,000 babies have been screened for inborn errors of amino acids and organic acids. Newborn urine samples are collected on filter paper by parents at 21 days of age. Voluntary compliance is good at 90%. Samples are analysed using a multiplex thin layer chromatography technique with a sequential-four reagent staining methodology. Two unidimensional ascending solvent migrations are performed for higher resolution. We analyse 500 samples per day totalizing 77,000 samples/year in 2008.

We screen for 25 disorders: (1) those causing severe clinical problems which necessitate immediate therapeutic intervention, such as urea cycle disorders and organic acidurias; (2) those necessitating surveillance and follow-up in metabolic disorders and transport disorders. Regarding urea cycle disorders, we have confirmed 18 cases of argininosuccinic aciduria, 5 citrullinemia type I, 3 citrullinemia type II, 4 hyperargininemias, and 1 Triple H syndrome. Concerning amino acid transport disorders, we found 146 cases of homozygous cystinuria and 1,002 heterozygous cystinuria, 15 Fanconi syndrome, 56 Hartnup disease and 71 dicarboxylicaminoacidurias. Our screening program is a dynamic model that has evolved throughout the years to screen as many treatable micromolecule disorders as possible using a rapid, simple, reproducible and low-cost methodology.

Treatment of urea cycle disorders: Are we making progress?

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Inborn errors of the urea cycle are estimated to affect 1:30,000 live born infants. Deficiency of the enzymes involved in this pathway lead to an inability to transfer nitrogen from ammonia to urea. In the classical neonatal form, disruption of the urea cycle is characterized by hyperammonemia, respiratory alkalosis and encephalopathy. Early recognition and acute stabilization of these disorders are essential in avoiding catastrophic outcomes. For long-term management, conventional approaches to treatment such as protein restriction, alternative pathways and product replacement have been in place for over 30 years. It is clear that our attempts at restoring nitrogen homeostasis are still lacking and morbidity remains high. Over the last 10 years, newer techniques including liver transplantation, chaperone therapy and gene therapy have been developed and show promise. This presentation summarizes outcome and therapy in inborn errors of the urea cycle to provide an overview of what has already been achieved and what needs to be accomplished.

Triple H syndrome: more questions than answers

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The triple H syndrome (OMIM #238970; hyperammonemia, hyperornithinemia, homocitrullinaemia) is an autosomal recessive disorder characterized by hepatocellular dysfunction, intermittent hyperammonemic encephalopathy, and spastic paraparesis, caused by a defect in ORNT1, involved in the transport of L-ornithine across the inner mitochondrial membrane, resulting in intra-mitochondrial accumulation of carbamoylphosphate and ammonium. The diagnosis is generally established by demonstration of elevated levels of ornithine, homocitrulline and ammonium in blood, and orotic acid in urine, and may be confirmed by specific mutation analysis. Studies done of 49 patients in Quebec, Canada, showed that the clinical phenotype varies significantly, even among patients homozygous for the same mutation (delF188) in *SLC25A15*. Treatment with dietary protein restriction and sodium benzoate prevented recurrence of hyperammonemic encephalopathy. However, it did not prevent the development of progressive spastic paraplegia, a prominent feature of most of the patients studied. Although some of the data suggested that intra-mitochondrial ornithine deficiency was an important cause of hyperammonemia, treatment with L-arginine, did not improve outcomes. The clinical phenotype is also different from that of lysinuric protein intolerance (OMIM #222700), another disorder of amino acid transport characterized by intra-mitochondrial ornithine deficiency. Part of the variability of the clinical phenotype in this population might be the result of polymorphisms of a second mitochondrial ornithine transporter, ORNT2, encoded by *SLC25A2*. The pathophysiologic mechanism of the hepatocellular dysfunction, cognitive deficits and spastic paraplegia observed in patients with the disease remains obscure.

Hypercitrullinemia type I and II: new insights and management strategies

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Hypercitrullinemia can be the hallmark of two distinct metabolic disorders, citrullinemia type I and II. The former is caused by deficiency in argininosuccinate synthetase (ASS) and the later by citrin deficiency. Both disorders can manifest in the neonatal period with hyperammonemia and liver dysfunction. Distinctions between these two disorders on clinical and biochemical grounds can be difficult. Until recently citrullinemia type II had been thought to be ethnically restricted to Asians. A key distinguishing feature has been that ASS

activity is deficient in liver, but normal in skin fibroblasts .Here we present a subgroup of Caucasian patients that showed a different enzymatic profile making this distinction even more challenging.

Five French–Canadian had citrin deficiency as a result of founder mutations in the gene SLC25A13, encoding citrin. In marked contrast to previous cases these patients have markedly reduced ASS activity in skin fibroblasts. Two of them had a presumptive diagnosis of ASS deficiency and developed liver failure and hypoglycaemia under a protein-restricted diet. However, they fully recovered after a high-protein diet once citrin deficiency was diagnosed. One of the adult experienced mood disorder that are difficult to associate clearly with citrin deficiency. No liver disease is observed in unmanaged adults.

Citrin deficiency could be more frequent than previously thought among Caucasians and its differentiation from ASS deficiency is mandatory in order to provide optimal treatment. The natural history of the disease is still unknown and demand further studies.

Transport disorders of amino acids: the Quebec experience

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The Quebec Network of Genetic Medicine began newborn mass screening for urinary amino acids in 1971 as part of a voluntary preventive medicine program. In the beginning, amino acids related to transport disorders were amongst the disorders targeted, along with urea cycle disorders and one organic acid, methylmalonic acid. The referral procedure is simple: after quantification of amino acids for abnormal cases detected by the Provincial Mass Urinary Screening Program at the Sherbrooke Medical Center, newborns are referred to four genetic clinical centers for diagnostic evaluation, confirmation, instigation of treatment if indicated, surveillance and follow-up. Five clinical amino acid transport disorders necessitate surveillance and counseling. We will review five main categories of inborn errors of transport disorders referred to the Service of Medical Genetics at CHUL in Quebec City from 1994 to 2008: (1) Cystinuria (homozygotes and heterozygotes patients); (2) Hartnup disease; (3) Dicarboxylicaminoaciduria; (4) Fanconi syndrome; and (5) Iminoglycinuria. Without taking into account cases of iminoglycinuria, the overall incidence for the aforementioned transport disorders is 1:1,290. A clinical picture of these cases, their evaluation and follow-up including biochemical data will be presented. The incidence of each disorder, usefulness and evidence-based indication for early diagnosis and treatment will also be presented. In conclusion, we emphasize the importance of screening for transport disorders of amino acids by a simple, inexpensive methodology such as thin layer chromatography, considering the potential impact on short and long-term morbidity.

The application of ultraperformance LC to the analysis of amino acids

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Amino acid analysis is an important tool in the study of a number of physiological processes. Changes in the concentrations of specific amino acids can result from various modifications at specific points in metabolic pathways. Quantitative amino acid analysis (AAA) has traditionally been performed using ion exchange chromatography with post-column ninhydrin derivatization—a time-consuming and high-maintenance methodology. In addition, it has become apparent that understanding many of these metabolic processes requires reliable analysis at lower concentrations than can easily be detected using more established methodologies.

We describe a new total application solution, for research use only, using UltraPerformance LC® in conjunction with tunable UV detection that addresses the shortcomings of the more conventional methods of AAA. This approach includes a well-characterized amino acid derivatization (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate), a thoroughly tested analytical method for analysis of the derivatized amino acids and compliance-ready software for acquisition and reporting.

We will present studies demonstrating both qualitative and quantitative aspects of the analysis including the linearity of response and sensitivity of each of the commonly measured amino acids in a physiological profile. Accuracy and precision for both standards and biological samples will be examined. Samples in these biological matrices will also be compared to existing standard methods. These quantitative studies will show the reliability and robustness of this solution for the analysis physiological amino acids.

Metabolomics and amino acids analysis of urine by proton magnetic resonance spectroscopy

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Proton magnetic resonance (^{1}H-NMR) Spectroscopy analysis of amino acids in urine and serum has been widely used for diagnosis and follow up of several inborn diseases. This aim was accomplished even on a "fingerprint" basis, without precise quali/quantitative analysis, in that many of such pathologies generate huge accumulation of amino acids and/or derivatives in the NMR spectrum so that very accurate quantitations were not required.

On the other hand, in more recent years, several studies have been devoted to the improvement of NMR techniques in order to increase accuracy of quantitation of urine components. Computer-aided estimation of up to hundreds of metabolites, including amino acids as well as robust pattern recognition methods of analysis of complex ^{1}H-NMR spectra are now available, so that research in metabonomics has come into the topic. Moreover, NMR "medium field" spectrometers up to 300 MHz, have become available at lower prices, easily affordable for any research and/or hospital institution.

Our efforts have been devoted to assess the utilization of ^{1}H-NMR analysis, in particular in estimating the normal amino acid content in urines, within the frame of ^{1}H-NMR multi-componental urinalysis, using and comparing 300 as well as 500 MHz spectrometers. For this purpose simple "home-made" computer programs have been used as opposed to more sophisticated ones. This study was run on a wide population of healthy subject urines, thus allowing us to calculate normal ^{1}H-NMR values of amino acids and derivatives with respect to creatinine content, estimated by ^{1}H-NMR as well.

Based on the obtained results, routine use of affordable medium field spectrometers can be proposed as a powerful tool for metabonomics and amino acid analysis of urine, and, similarly, of other biological fluids such as serum, cerebro-spinal fluid and others as well.

L-arginine and L-citrulline: production technology in genomic era

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L-Arginine, a semi-essential amino acid, has lately attracted considerable attention because the amino acid has been shown to be a precursor to nitric oxide (NO), a key component of endothelial-derived relaxing factor. Because of L-arginine's NO-stimulating effect, the amino acid helps, for example, to relax and dilate blood vessels, and thus can be utilized in numerous clinical areas. On the other hand, L-citrulline, a precursor of L-arginine biosynthesis, is also an important amino acid for our health since it is a source of endogenous L-arginine in the body. These two L-amino acids are produced by fermentation using classically derived regulatory mutants of *Corynebacterium glutamicum*, a representative amino acid-producing microorganism.

We previously developed a methodology to reengineer a more efficient producer using knowledge regarding the mutations that have accumulated over years of industrial strain development. In this methodology, biotechnologically useful mutations identified through the genome analysis of classical mutants are systematically introduced into the wild-type genome in a pinpointed manner, thus allowing creation of a defined mutant that carries only useful mutations. Furthermore, with the accumulated knowledge on mutations relevant to production, it becomes possible to combine positive mutations derived from different lines of classical producers in a single wild-type background. Such an advanced approach has recently led to an impressive result in production of L-arginine and L-citrulline by *C. glutamicum*. The procedure and impact of this reengineering methodology are described here.

Acknowledgments: This work has been arranged mainly based on the research fruits conducted with my co-workers, S. Mitsuhashi, J. Hayashi, K. Tanaka of Kyowa Hakko Bio Co.

Molecular ontology of essential amino acid transport

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18 L-amino acids, ahiral glycine, and imino proline comprise the proteinogenic foundation of Life. They also serve as principal carriers of fixed nitrogen between cells of organisms and between organisms in nutrient chains of the Biosphere. Small amino acids are ubiquitously biogenic, but syntheses of the $\sim$10 most metabolically expensive in bacteria and plants large aliphatic and aromatic amino acids were lost in eukaryotic heterotrophs, which must transport these essential substrates against thermodynamically unfavorable permeability and gradients. Surprisingly, the analysis of the anticipated transport network for essential amino acids is fragmented in mammals and virtually absent in the rest of the animal Kingdom. Using bioinformatics and molecular biology approaches we have identified, cloned, and heterologously expressed a representative set of NAT-SLC6s (Nutrient Amino acid Transporters of the Neurotransmitter Sodium Symporter family, NSS, a.k.a. SNF and SLC6) from selected invertebrate model organisms. In addition to mammalian B0 system-like broad spectra transporters, we have identified transporters with selectivity for phenylalanine/phenol-branched, tryptophan/indole-branched, or methionine/cysteine/sulfur-containing substrates, together absorbing the most underrepresented essential amino acids. The NATs are localized ubiquitously but over-expressed in the apical membrane of the posterior gut and specific neurons of the brain. The consensus of identified biological characteristics implies that these NATs evolved to transport essential and conditionally essential amino acids. Expansions of NATs' phenotypes and their shaping of metazoan transport mechanisms and metabolism of essential amino acids will be discussed.

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Low urinary serine in a group of subjects screened for inherited metabolic disorders

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Objectives: L-Serine (Ser)-deficiency disorders comprise a new group of potentially treatable neurometabolic diseases caused by defects in the Ser biosynthesis, associated with congenital microcephaly, psychomotor retardation and seizures. Low plasma (or combined with low urine) Ser levels have also been demonstrated in other conditions. Isolated absence or very low Ser in urine without any abnormalities in the plasma, however, has never been reported.

Methods: Our four decades practised screening strategy for detection of amino acid abnormalities starts with 2D TLC on cellulose and follows (when necessary) with quantitative analysis (cation-exchange chromatography, CZE and RP HPLC were used in course of time).

Results: No Ser has been detected on TLC aminogram from urine of several subjects (often hypotonic, mentally or motorically retarded with seizures and other symptoms). Contrary to normal plasma concentrations, very low urinary Ser levels (in some cases namely those of D-Ser) were confirmed by quantification. Concentrations of other amino acids, organic acids and saccharides did not deviate from reference ranges. Cerebrospinal fluid was not available. Gastrointestinal or renal problems were not found; effect of therapy is not probable. More then 30 of such patients including two adults (related partners) have been detected in the last 5 years; reasonable elucidation still fails.

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Amino acids

The influence of disposal technology obtained with alkaline treatments on D-amino acid content of slaughter waste

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In our experiment change in D-amino acid content of alkaline treated slaughterhouse waste material was examined. Originally, the aim of the treatments was to find out whether it is possible to destroy

microorganisms and other harmful materials possibly being present, and by this to make meat flour suitable for foddering purpose. The treatments were carried out with sodium and potassium hydroxide solution for 2, 3 and 6 hours at 135, 150 and 153°C. D-Asp, D-Glu-, and D-Trp content was determined using a *Hitachi Merck LaChrom* HPLC, while D-allo-Ile content was determined by an Ingos amino acid analyzer. In case of Trp the hydrolysis was carried out with 3 M *p*-toluenesulfonic acid in the presence of 3-indolyl propionic acid at 110 ± 2°C. For the rest of the amino acids 6 M hydrochloric acid was applied for the hydrolysis at the same temperature. Summarized, it can be said that due to the temperature and alkali combinations applied 40–50% of aspartic acid, tryptophan and isoleucine converted into the D-isomers. Even though other parameters of the obtained hydrolyzed products meet the requirements of the modern foddering, it should be taken into consideration that most of the amino acids undergo a complete racemization.

Trace determination of thioglycolic acid and thiosulphate using novel inhibitory kinetic spectrophotometric method

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Sulphur containing compounds such as sodium thiosulphate (STS), thioglycolic acid (TGA) inhibit the rate of cyanide substitution by nitroso-R-salt (NRS) in hexacyanoruthenate(II) catalysed by Hg(II) ions due to their strong binding tendencies with Hg(II) catalyst. This inhibitory effect of sodium thiosulphate and thioglycolic acid is used as the basis for their determination at micro levels. The reaction was followed spectrophotometrically at 525 nm (λ_{max} of $[Ru(CN)_5NRS]^{3-}$ complex) under optimised reaction conditions at $8.75 \times 10^{-}$ M $[Ru(CN)_6^{4-}]$, 3.50×10^{-4} M [NRS], pH 7.00 ± 0.02, ionic strength (μ) 0.1 M (KCl) and temp 45.0 ± 0.1°C. The modified mechanistic scheme is proposed to understand the inhibition caused by sulphur containing compounds (STS and TGA) on Hg(II) catalyzed substitution of cyanide by NRS in $[Ru(CN)_6]^{4-}$. The range of analytical concentration of inhibitor depends upon two factors; the amount of Hg(II) catalyst present in the indicator reaction and the stability of the Hg(II)–inhibitor complex under consideration. Under optimum conditions STS and TGA have been determined in the range of 0.98–7.0×10^{-6} M and 0.30–7.0×10^{-6} M. The detection limits for STS and TGA were found to be 3.0×10^{-7} M and 4.5×10^{-7} M, respectively.

Spectroscopic study on interaction of human lactoferrin with lomefloxacin at physiological condition

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Lactoferrin (Lac) is an iron-binding protein that is closely related to the plasma iron-related protein transferring. It is a 673 amino acid Fe^{3+} binding glycoprotein. It is a single-chain glycoprotein with a molecular weight of 80 kDa. Quinolones are a group of compounds widely used as broad-spectrum antibacterial agents. They derived from nalidixic acid. Lomefloxacin (LMF) is one of the synthetic antibacterial in the third generation which exhibit high activity against a broad spectrum of gram-negative through inhibition of their DNA gyrase. It is used to treat respiratory tract, urinary tract and skin structure infections. Lactoferrin and lomefloxacin were dissolved in Tris-buffer, at room temperature. The concentration used of Lac was 6.25×10^{-9} mol/l. Fluorescence-quenching measurements have been widely used to study the interactions of drugs with proteins. This method can reveal accessibility of quenchers to protein fluorophore, help to understand protein binding mechanisms to compounds and provide a clue to the nature of the binding phenomenon. The excitation wavelength was set at 280 and 295 nm respectively. Wavelength of 280 nm excites the tryptophanyl and tyrosyl residues while 295 nm excites only the tryptophanyl residues. When excited at 280 nm, Lac gives rise to emission spectra in the range of 290–370 nm with λ_{max} at 328 nm. Under the similar condition, the fluorescence has been observed for LMF alone in the range of 350–550 nm. Further addition of LMF into Lac solution resulted in the decrease of the protein fluorescence intensity. The fluorescence quenching indicated the binding of LMF to Lac resulted in microenvironment change of tryptophan and tyrosine residues. The fluorescence quenching behavior in the system of LMF and protein could be analyzed by the Stern–Volmer equation. The K_{sv} values of Lac-LMF complex at different pH (5.4, 6.4, 7.4, 8.4 and 9.4) have been calculated and compared with together. Circular dichroism spectroscopic technique showed that the secondary structure of Lac was been changed on interaction with LMF.

Research on hydrolysis technology and reaction kinetics for amino acids production from fish waste in sub-critical water

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The hydrolysis technology and reaction kinetics of amino acids production from fish waste in subcritical water by adding carbon dioxide has been conducted. The effects of reaction temperature, time, amount of CO_2 and pressure (adding N_2) on the yield of amino acids were investigated respectively. The experiment results show that the optimum hydrolysis conditions for amino acids production from fish waste are as follows: temperature 513 K, amount of CO_2 5.0 MPa, amount of N_2 3.0 MPa and reaction time 18 min. Under this condition, the yield of amino acids can reach 31.53%. The chemical reaction kinetics experiment results indicate that the total velocity constants of fish waste hydrolysis in sub-critical water are 0.036, 0.071, 0.14, 0.33 at 493, 513, 533, 553 K respectively with the amount of CO_2 of 4.9 MPa and pressure (adding N_2) of 3.0 MPa. The reaction kinetics order is 1.28, active energy is 82.75 kJ/mol and the pre-exponential factor is 1.97×10^7.

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Bioinformatics

Rationalizing the selection of empirical reference data to benchmark B-cell epitope prediction for peptide-based vaccine design

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B-cell epitope prediction, which supports the design of peptide-based vaccines, has largely been benchmarked using empirical reference data acquired by probing antiprotein antibodies for cross-reactivity with peptide antigens. The results thus obtained generally suggest underperformance of available methods for B-cell epitope prediction, notably those based on sequence profiling with amino acid propensity scales; however, these results are of uncertain relevance to the design of peptide-based vaccines, for which the appropriate empirical reference data are those acquired by probing antipeptide antibodies for cross-reactivity with protein antigens. Furthermore, closer consideration of peptide-based immunogens and the antibody responses that they elicit reveals potential complications in the benchmarking process, which are appreciated in relation to the phenomenon of immunodominance, the impact of immunization with peptide-protein conjugates, and the distinction between genuine and apparent cross-reactivity of antipeptide antibodies with protein antigens. The caveats disclosed by this analysis highlight the importance of judicious selection of empirical reference data to benchmark B-cell epitope prediction for peptide-based vaccine design. The selection process should favor data acquired using antibodies raised against unconjugated peptides that closely resemble segments of their cognate protein antigens in terms of both amino acid sequence and distribution of electrical charge; in addition, evidence of antibody-modulated protein activity could be required to confirm positive cross-reactivity as genuine rather than apparent, and the absence of detectable binding of antibody to protein in a fluid-phase immunoassay could be required to confirm negative cross-reactivity (thus avoiding the problem of adsorption-induced inaccessibility to antibody that arises in solid-phase immunoassays).

Interpro: using protein signatures to decipher the function of the human proteome

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The human proteome currently stands at 25,635 proteins. Of these, 20,332 are curated in the UniProtKB/SwissProt database. For the remainder (66,734 UniProtKB/TrEMBL entries, including redundancies), there is a pressing need for elucidating functional information that goes beyond the capabilities of experimental work alone. Computer algorithms that take into account sequence identity, structural similarity and phylogenetic tree distribution are invaluable for protein function prediction.

InterPro (http://www.ebi.ac.uk/interpro/) is an open-source protein resource combining ten signature databases (PROSITE, PRINTS, PFAM, PRODOM, SMART, TIGRFAMs, PIR-SUPERFAMILY, PANTHER, GENE3D, SUPERFAMILY) into a powerful diagnostic tool. InterPro provides comprehensive annotation on function, structure, splice variation, taxonomic distribution and hierarchical classification of proteins from all organisms. Protein signatures model amino acid conservation in known families or domains, then use this information to predict the classification/features of uncharacterised proteins. With $\sim$60,000 signatures, InterPro provides annotation for $\sim$80% of all UniProtKB sequences, therefore even SwissProt-curated proteins can gain additional annotation. By linking related signatures together, InterPro places them within a hierarchical classification, reflecting underlying evolutionary relationships. As such, one can address issues such as the co-evolution of domains, and the functional divergence of proteins based on domain composition.

InterPro can be used to analyse the human proteome as a whole, providing information on the occurrence of specific domains, and the distribution of functional roles (e.g. enzymatic, structural…). InterPro is a useful source of experimental targets, highlighting which groups of related proteins lack structural or functional information. With its multi-layered approach, InterPro is a valuable resource for biologists and bioinformaticians alike.

Bioinformatics combined with epitope extraction technique: Excellent tool for structural analysis of potential allergenic epitopes

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Bioinformatics is the branch of life science concerned with application of information technology to the field of molecular biology. The immune system represents a large scientific field where bioinformatics make a large impact. The immune system reacts to foreign protein segments called epitopes and a major task is to develop methods for their prediction and identification usable eventually in vaccine design and diagnosis.

This study was focused on identification of allergenic epitopes of food allergens. We applied bioinformatics tools for structural study of the ovalbumin fragment 371–382, which was identified as potential allergenic epitope by using microfluidic magnetic force-based epitope extraction technique (J Chromatogr A 1206:64–71, 2008). The location and accessibility of this fragment were determinate by aligning linear peptide sequence to the 3D allergen structure.

Position of the fragment in 3D structure of ovalbumin shows that residues 377–382 are part of the C-terminal anti-parallel β-sheet in the core of the protein structure and residues 371–376 form a loop connecting with no particular secondary structure, which is exposed to the protein surface. Detailed analysis was also performed to quantify the solvent accessibility of individual residues in the peptide. Observed results confirmed partial accessibility of target fragment to immune system, associated with potential induction of allergic reaction. This approach is suitable for detection of significant immunogenic epitopes for subsequent application in clinical practice.

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Attempt of stoichiometric extension of amino acid metabolism-related model metabolic network

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Whilst genome information is available in many organisms, there are genes function of which has not been elucidated, indicating that enzyme reaction as novel gene function may be found. We have proposed to extend metabolic network based on stoichiometry of enzyme reaction as a strategy to find candidates of new enzyme reaction. The resulting extended network is composed of the original real reactions and hypothetical reaction to be considered as a candidate of new enzyme reaction. This presentation shows concept and status of this method and attempt to apply this to model metabolic network related to amino acid metabolism.

PROFESS: *pro*tein *f*unction, *e*volution, *s*tructure and *s*equence

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The proliferation of biological databases and the easy access enabled by the Internet is having a beneficial impact on biological sciences and transforming the way research is conducted. Current biological databases present many types of problems, from poorly defined user-interfaces to limited and error-prone search options. We introduce the *pro*tein *f*unction, *e*volution, *s*tructure and *s*equence (PROFESS) database, a genome biology database system to assist in the functional and evolutionary analysis of the abundant number of novel proteins generated by sequencing and structural projects. The database will aid in the study of protein evolution and annotation by linking organism with protein orthologous function, protein structure and classification, gene ontology, protein interactions, bound ligands and metabolic pathways, etc. Our database is designed to be versatile and expandable and will not confine analysis to a pre-existing set of data relationships. A fundamental component of this approach is the development of an intuitive functional-style query system that incorporates a variety of similarity operators capable of generating data relationships not conceived during the creation of the database. *PROFESS* currently integrates over thirty system biology database tables and is implemented using MySQL and AJAX. *PROFESS* is freely available online at http://cse.unl.edu/~profess/.

Comparison of NRC-2001 model with DVE/OEB system in the prediction of protein value from different types of bioethanol co-products (DDGS)

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The NRC-2001 model is a TDN-based model popularly used in North America. The DVE/OEB system is a Non-TDN based model. Both models introduced the new concepts of: (1) metabolizable protein, defined as true protein that is absorbed by the intestine, and contributed by ruminally undegraded feed protein, ruminally synthesized microbial protein, and endogenous protein from rumen; (2) Rumen protein degradation balance reflects the difference between the potential microbial protein synthesis based on ruminally degraded feed protein and that based on energy available for microbial fermentation in the rumen.

The objective of this study was to compare the TDN-based NRC-2001 Model with the non-TDN based DVE/OEB system in the prediction of protein degradation balance and protein supply to dairy cattle from 17 different types of Dry Distillers Grains with Solubles (DDGS) and feedstocks.

Comparisons were made in terms of: (a) ruminally synthesized microbial protein (AMCP); (b) ruminally undegraded feed protein (ARUP); (c) endogenous protein (AECP); (d) total metabolizable protein (MP); and (e) degraded protein balance (DPB).

The modelling results showed that there were no correlation in AMCP estimation ($P > 0.05$; $R = 0.28$), but strong correlations in the prediction of AECP ($P < 0.001$, $R = 0.72$), ARUP ($P < 0.001$, $R = 1$), MP ($P < 0.001$, $R = 0.99$) and DPB ($P < 0.001$, $R = 0.97$) between the NRC-2001 and DVE/OEB system.

However, using the DVE/OEB system, the overall average microbial protein supply based on available energy was 5% higher, endogenous protein 17% higher, ruminally undegraded feed protein 10% higher, and the truly absorbed protein in the small intestine was 4% higher than that predicted by the NRC-2001 model. There was not much different in degraded protein balance prediction (only 0.3% difference)

No much difference was also found in the prediction of the degraded protein balances, which was only 0.3% higher than that estimated from the NRC-2001 model.

These differences are due to factors used in prediction in the two models, although both are based on similar principles. This indicates that further refinement is needed for a modern protein evaluation and prediction system.

Biology and microbiology

Molecular mechanism of growth inhibition of *Escherichia coli* by L-cysteine

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Redox equilibrium (a balance between oxidized and reduced states) governs many essential biological processes in the cell. It is well known that *Escherichia coli* cells undergo growth inhibition by L-cysteine, which is a serious problem in L-cysteine fermentation. In our previous report, the *dsbA*-disrupted *E. coli* cells showed hypersensitivity to L-cysteine and DTT. Here, we found that the disulfide isomerase DsbA is reduced by exogenous L-cysteine, and then the outer membrane protein OstA, which is essential for growth of *E. coli*, was also accumulated in a reduced state. In contrast to the reduced DsbA, L-cysteine is oxidized to L-cystine, and is taken up from the periplasm to the cytoplasm of *E. coli* cell by the inner membrane protein FliY that is most similar to the L-cystine binding protein BspA from *Lactobacillus fermentum*. We concluded that the DsbA-DsbB-ubiquinone oxidation system might eliminate reductive stress by oxidizing exogenous reductants and also play an important role in redox homeostasis in the cytoplasm. Furthermore, we showed that *E. coli* cells were tolerant to hydrogen peroxide, since the YdeD protein transports intracellular L-cysteine into the periplasm. We propose that the L-cysteine/L-cystine circulating system is essential for the protection of *E. coli* cells from reductive or/and oxidative stress (es).

Overexpression, purification and characterisation of a short chain alcohol dehydrogenase (HpSCADH) from *Helicobacter pylori*

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A short chain alcohol dehydrogenase is one of three alcohol oxidising enzymes identified in the annotated genome of *H. pylori* 26695. Two of these were previously characterised in this laboratory. They were found to have broad specificity for a range of aromatic aldehydes/alcohol substrates. The short chain alcohol dehydrogenase (HpSCADH) is of particular interest since its characterisation will provide a complete picture of aldehyde reducing enzymes in this bacterium. Aldehyde detoxification in *H. pylori* is of interest since toxic aldehydes have been implicated in the pathogenesis of *H. pylori* related damage to the gastric mucosa. The HpSCADH gene was cloned and expressed in *Escherichia coli* as a His-tag fusion protein, and purified using nickel chelate chromatography. A protocol to purify a stable preparation of this protein was devised. The enzyme is a monomer of approximately 29 kDa. HpSCADH can function over a pH range of 6–9, and has temperature optimum of 37°C. Preliminary data show that this enzyme has a preference for NAD^+ over $NADP^+$ and that it is active with a range of aliphatic alcohol substrates. HpSCADH was classified as classical short chain alcohol dehydrogenase based on the sequence alignment studies. Application of this enzyme in asymmetric synthesis will be explored.

A culture medium of chitinolytic bacteria evaluated by colloid chitin azure

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Chitin and its derivatives are widely used as biomedical materials on account of their versatility and biocompatibility. Chitinases are enzymes that produce chito-oligosaccharides from chitin. The assay of chitinase activity is difficult, because few appropriate substrates are available. In this study, we develop an efficient and low-cost chitinase assay using colloidal chitin azure. The assay feasibility is evaluated and compared with traditional assays employing colloidal chitin and chitin azure. We found that the optimum pH for determination of chitinase activity using colloid chitin azure was pH 8. The method was sensitive, and the assay was complete within 30 min. When the assay was used to measure chitinase activities produced by two strains of chitinolytic bacteria, BCTS and AS1, it was shown that cultivation in BHS medium caused AS1 to secrete a higher level of chitinase than was secreted when the bacterium grew in other media. In summary, the effect of culture medium can be evaluated with colloid chitin azure.

Enzymatic hydrolysates from marine organisms of the Barents sea

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The cycle of biochemical and technological studies on search of objective criteria for assessment of marine hydrobionts hydrolysis efficiency and choice of optimum protein raw materials is presented. A number of original technological decisions for production of enzymatic protein hydrolysates for microbialogic, nutritional and feed assignment is offered.

Various waste of a marine catch and processing, and also invaluable trade kinds of hydrobionts of Barents sea are considered as potential raw materials.

In work the characteristics of raw materials and prepared hydrolysates, the approach to selection of enzymatic preparations with definition of optimum hydrolysis conditions are shown. Effect of various factors (pH, temperatures, concentrations of enzyme) on kinetics and degree of hydrolysis of protein-containing raw material is considered in detail.

The kinetics of change of protein hydrolysates contents during incubation is analyzed by the exclusion chromatography HPLC and electrophoresis, and also by definition of amino acid composition and the contents of free amino acids in a hydrolysate.

On the basis of results of these researches the criteria of assessment for protein hydrolysis efficiency with application of such methods as gravimetric, spectral, gel-filtration, contents definition amine and nonprotein nitrogen, free amino acids are offered.

Possibility of usage for hydrolysis low-cost proteolytic preparation from hepatopancreas of King crab and the enzymes extracted from it, instead of commercial enzymatic preparations, is convincingly proved.

In the report results of experimental approbation of the prepared enzymatic protein hydrolysates as part of microbialogic media, feedstuffs for young growth of chickens and starting feedstuffs for salmon are fetched.

Medicine

Hemorphin-7 peptides metabolism in diabetes

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Low circulating VVH7-like immunoreactivity (VVH7 i.r) level was amazingly observed in human diabetic sera. Here, we examined the impact of diabetes type, clinico-biological features and metabolic control on circulating VVH7 i.r level in this disease. ELISA test was used to measure VVH7 i.r in sera of 120 diabetic patients (type 1 diabetes in 64, type 2 diabetes in 56). Three enzymatic tests were also applied to determine serum cathepsin D (CD), dipeptidyl peptidase IV (DPP-IV) and angiotensin converting enzyme (ACE) activities. A subgroup of 24 type 1 diabetic patients negative for microalbuminuria and hypertension were submitted to an ambulatory blood pressure monitoring to evaluate

the relationship between VVH7 i.r level and blood pressure parameters. The mean serum concentration of VVH7 i.r was drastically reduced in diabetic patients (0.91 ± 0.93 μmol/l vs. 5.63 ± 1.11 μmol/l in controls) ($P < 0.001$). A negative correlation between VVH7 i.r level and daytime diastolic blood pressure existed in type 1 diabetic patients. There was no association of low VVH7 i.r with either type of diabetes or HbA1c level. An increase of cathepsin D activity was found in serum of diabetic patients compared to controls (0.47 vs. 0.15 U/ml respectively) whereas DPPIV activity was significantly decreased in diabetic sera (50.81 vs. 282.10 U/l respectively). Diminution of VVH7 i.r in sera of diabetic patients was confirmed but still remained unexplained. Relationships between higher systolic blood pressure and decrease of VVH7 i.r reinforce the need to investigate this pathway in this disease to elucidate its role in macro- and micro-angiopathy.

A pH-sensitive colon targeted oral drug delivery system using insulin nanoparticles employing chitosan with different molecular weights and Eudragit L100-55

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The objective of the present study was to develop a novel pH-sensitive nanoparticle system that was suitable for entrapment of hydrophilic insulin. Eudragit L100-55 was used as a negatively charged polymer for preparation of four insulin nanoparticles. Chitosan was incorporated as a positively charged material, and consisting of three different molecular weights. The mean diameters were in the range 600–700 nm, and loading efficiencies from 40–80%. Higher encapsulation efficiency was obtained with nanoparticles prepared with high molecular weight of chitosan. Different molecular weights of chitosan were the factor which influenced the nanoparticles significantly. The results also demonstrated that Eudragit L100-55 and chitosan can be successfully used for colon targeted delivery of insulin and the formulation can be adjusted to deliver drug at any other desirable site of the intestinal region of the GI tract. When increasing the molecular weight of chitosan, the release appeared more controlled and prolonged up to 8 h. The modeling data indicated that the release kinetics of insulin was nonlinear, and during the release process, the nanoparticles showed a polynominal swelling. This new pH-sensitive nanoparticle formulation using chitosan and Eudragit L100-55 polymer may provide a useful approach for entrapment of hydrophilic polypeptides like insulin.

Biomarker development for personalized medicine

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Cancer is a diverse disease, and the present clinical and pathological diagnostic modalities have obvious limitations. The next level of molecular diagnostics using novel biomarkers has been desired to optimize the existing therapeutic strategy. As proteome is a functional translation of genome, directly regulating cancer phenotypes, proteomic approach should be effective in biomarker development. To investigate proteome contents in surgically resected tumor tissues, we established one of the largest proteomic laboratories at the National Cancer Center. Our proteomics system is based on the application of two-dimensional difference gel electrophoresis (2D-DIGE), where protein samples are labeled by high sensitive fluorescent dye before gel electrophoresis. Our proteomic studies using more than 1,000 tissues with clinico-pathological data resulted in the identification of proteins corresponding to the response to treatment, early recurrence, and short survival period in lung cancer, esophageal cancer, colorectal cancer, liver cancer, and soft-tissue sarcoma. Such proteins were considered as biomarker candidates for personalized medicine. To establish a clinical examination using the identified proteins, we developed monoclonal antibodies. Immunohistochemical validation studies in the different hospitals confirmed the predictive performance of the biomarker candidates. The recent clinical applications of our biomarkers will be demonstrated, and the strict limitation and solution for the present proteomics approach will be discussed in the presentation.

Encapsulation of insect sex pheromone via simple and complex coacervation of gelatin

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With insect sex pheromone molecules as core material, natural polymers gelatin (GE) and acacia gum (AG) as wall materials, microcapsules, aiming as **s**prayable environment-friendly pesticide, were prepared through simple coacervation of GE and complex coacervation of GE and AG. Dodecanol (DOC), and oleyl acetate (OA) as model molecules for insect sex pheromone, were encapsulated to provide credible information on the encapsulation, loading and the release control of insect sex pheromones. The optimum experimental conditions in simple and complex coacervations were first explored. Adequate amounts of ethanol and sodium sulfate, both as coacervation inducer in GE simple coacervation, were determined. The properties of microcapsules prepared using the two coacervation inducers were investigated. Ethanol induced simple coacervation resulted in capsules with a lower pheromone molecule encapsulation compared with those by sodium sulfate. For GE-AG complex coacervation, optimum pH for maximum yield was determined to be 4.0. Pheromone molecule encapsulation in this complex coacervation was higher than those in simple coacervation. And DOC encapsulation was significantly enhanced with increase in crosslinking of wall materials. Results of the release of the encapsulated pheromone molecules at constant temperature (35°C) and relative humidity (50%) revealed that all samples from simple coacervation reached their final release within one week, whereas microcapsules from complex coacervation manifested a two-step release profile, i.e. a quick start followed by a steady state release till completion. With increase in wall material crosslinking, release of the pheromone molecules was slowed down and the constant release was prolonged.

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Mutation detection of BRCA1 gene from Malaysian breast cancer patients by denaturing high performance liquid chromatography (DHPLC)

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Introduction: Breast cancer is a common malignancy affecting women globally. In Malaysia, it constitutes 31% of all newly diagnosed female cancer cases. Approximately 50% of the cases occur in women under the age of 50 years. A proportion of these cases may be attributable, at least in part, to genetic components. Women who carry BRCA1 mutations have a probability of about 80% for developing breast cancer, and 40 to 60% for developing ovarian cancer during their lifetime.
Aim of study: To screen for germline mutations in BRCA1 gene, in breast cancer patients from East Coast Malaysia with either early-onset breast cancer (at age $\leq$ 45 years) or a positive family history of breast cancer.
Materials and methods: Collaborating with the IIUM Breast Centre and the Surgery Department at Hospital Tingku Ampuan Afzan (HTAA), Kuantan, 40 peripheral blood samples were collected from women with either early-onset breast cancer or a positive family history of breast cancer. Mutations were detected in exon 11 of BRCA1 gene by dHPLC.
Results: Mutations were detected in 12.5% (5/40) of the patients. Three of them were Chinese, one Indian and one Malay.
Conclusion: This is a preliminary report on germline BRCA1 Exon11 mutation screening. Further study is going on to screen for mutations by protein truncation test and DNA sequencing.

Heat shock protein 90 regulates STAT1 nuclear translocation and IL-6 release in angiotensin-induced vascular cell hypertrophy

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Heat shock protein 90 (Hsp90), one of the most abundant proteins in the eukaryotic cells, is essential for cell survival. Cellular hypertrophy which is characterized increased protein synthesis is critical in vascular remodeling associated with hypertension, atherosclerosis, and restenosis. Angiotensin II plays a critical role in the cellular hypertrophy. However, the role of Hsp90 is largely unknown in angiotensin II-induced vascular smooth muscle cell (VSMC) hypertrophy. In this study, we investigated the role of Hsp90 in angiotensin II-induced VSMCs hypertrophy. We observed the [^{3}H]leucine incorporation level and protein/DNA content were increased by angiotensin II in a concentration-dependent manner in the VSMCs. During that period, nuclear translocation of STAT1 and IL-6 secretion were also increased. Interestingly, treatment with the Hsp90 inhibitor geldanamycin (GA) significantly abolished the angiotensin II-induced hypertrophy in VSMCs. GA also suppressed nuclear translocation of STAT1 and extracellular release of IL-6 in angiotensin II-stimulated VSMCs. Furthermore, treatment of IL-6-neutralizing antibody decreased angiotensin II-induced VSMCs hypertrophy. However, intracellular delivery of Hsp90 significantly restored angiotensin II-induced hypertrophy in VSMCs. These results indicate that angiotensin II-induced cellular hypertrophy via STAT nuclear translocation following IL-6 secretion and this pathway can be regulated by modulating Hsp90 activity. So, Hsp90 is another target protein for cardiovascular disease, especially vascular remodeling.

Apparent lack of alteration in the expression of the scaffold protein PICK1 in the spinal cord a rodent model of amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the progressive and selective loss of motoneurons. Glutamate-dependent excitotoxicity is considered as a key process in the progression of the disease. Indeed, high levels of glutamate are detected in the cerebral spinal fluid of ALS patients, consistent with a selective loss of glutamate transporter EAAT2 in their spinal cord and motor cortex. Furthermore, a motoneuron specific defect in the mRNA encoding the ionotropic glutamate receptor AMPA subunit GluR2 is thought to enhance motoneuron sensitivity to glutamate stimulation.

The protein interacting with C kinase 1 (PICK1) is a PDZ domain containing protein involved in the cell trafficking of several partners implicated in the control of glutamate transmission, including GluR2, EAAT2b and serine racemase. The aim of this study is to characterize the expression of PICK1 expression in a transgenic rat model of ALS ($hSOD1^{G93A}$).

Quantitative RT-PCR analyses performed on spinal cord samples at different stages (presymptomatic, onset and end-stage) failed to evidence any alteration of PICK1 mRNA expression in the transgenic rats as compared to wild type animals at the same age. Considering the massive loss of neurons and extensive gliosis in the spinal cord of the diseased animals, this result raises questions, as PICK1 expression is essentially documented in neuronal cells in the CNS. Therefore, further immunohistochemical analyses on spinal cord sections should help to clarify the cellular and subcellular localization of PICK1 in the spinal cord and to examine possible alteration in ALS.

Characterization, biodisponibility and biological effects of polypeptide fragments from the pericellular matrix in tumour progression

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Cellular microenvironment acts as a reservoir of signals which modulate cell behaviour and the progression of tumour. Among pericellular matrix components, matrix proteins, including collagens, and recruited plasma proteins play a central role through modules whose interactions with membrane receptors activate or inhibit signalling pathways involved in proliferation, apoptosis, migration, adhesion and differentiation of cells. These modules, which can be cryptic, are exposed after conformational changes of the parent molecules, particularly through proteases that cleave these molecules in polypeptide fragments. Interplays with other matrix components, chiefly glycosaminoglycans (GAGs) which immobilize and/or present these bioactive polypeptides to their receptors constitute a second step of regulation of their biodisponibility. Depicting these mechanisms which occur during pericellular matrix remodelling in tumour development should allow (1) to better understand the role of the microenvironment in tumour progression; (2) to identify putative biomarkers and drug targets, less toxic and more specific that current conventional chemotherapies. The project aims at studying bioactive modules which regulate tumour cell (proliferation/apoptosis) and endothelial cell (angiogenesis) behaviour, based on the analysis of collagen 18 and haemoglobin derived fragments as paradigms; furthermore, new bioactive modules will be identified and the role of proteases and GAGs in their biodisponibility will be studied.

Walker tumour's proteolysis-inducing factor affects the vero cells activity in culture and these alterations can be modulated by leucine

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Cancer cachexia is characterized by severe body protein wasting. Studies investigated this condition by evaluating skeletal muscle protein turnover; however, cachexia can also be related to non-skeletal muscle protein depletion. Factors produced by tumour play a critical role in wasting during cachexia since host protein degradation is affected by proteolysis-inducing factor (PIF) produced by tumour. Amino acid leucine is an important cellular signalling, by decreasing ubiquitin-proteasome pathway activity in Vero cells and in skeletal muscle cells, and by improving total protein synthesis in tumour-bearing rats. In this study, were investigated the effect of leucine in Vero cells exposed to a PIF-like protein purified from ascitic fluid of Walker-256 tumour-bearing rat, named Walker factor (WF), evaluating cell viability, alkaline phosphatase enzyme and proteasomal, lysosomal and calcium-dependent proteolytic pathways. WF has no cytotoxic effect in Vero cells after 24 h incubation, independent of leucine exposition. At higher WF concentration (15, 20, and 25 μg/mL) chymotrypsin-like (proteasomal pathway) and cathepsins B and H (lysosomal pathway) activities were increased; calcium-dependent pathway (calpain activity) did not change compared to the control cells, besides trended to decrease at lower WF (1, 3, and 5 μg/mL) and to increase at higher WF concentration; similar pattern was presented by alkaline phosphatase enzyme, which suggests cellular activity. Adding leucine (25 and 50 μM) 2 h previously to the 24 h WF exposition, all the proteolytic systems activities were decreased and alkaline phosphatase trended to increase. Taken together, these results strongly suggest a modulatory effect of leucine under the WF effects on Vero cells activity.

Effects of peripheral glutamate receptors on tumor cell growth: liaisons of neurotransmitters and body functions

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Glutamate receptors are not only crucial for excitatory neurotransmitter signalling in the central nervous system. In the last years it has been unequivocally proven that glutamate receptors are also expressed in peripheral non-excitable cells. Furthermore, it became apparent that peripheral glutamatergic signalling differentially modifies the proliferation, metabolic cell activity and morphology of tumor cells. We could show that ionotropic glutamate receptor reagents affect the proliferation of human histiocytic lymphoma-derived U937 cells depending on the external glutamate milieu. In addition, treatment with the competitive AMPA/kainate receptor antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) resulted in increased enzymatic activity of mitochondrial dehydrogenase combined with enlarged mitochondria in U937 cells. As to metabotropic glutamate receptors (mGluR), we found that several subtype-specific, non-competitive mGluR1 antagonists decreased the cell growth of different human melanoma cell lines. Electron microscopic analyses revealed specific morphological changes following administration of mGluR1 antagonists. Furthermore, we observed that the combined administration of an mGluR1 antagonist and an established chemotherapeutic drug resulted in a stronger cytostatic effect than either treatment alone. To confirm mGluR1 expression in melanomas we carried out RT-PCR analyses and found that all melanoma cell lines tested expressed mRNA for mGLuR1, whereas it was not detected in normal melanocytes. Even though the mGluR1 antagonists interact with a common allosteric site for non-competitive mGluR1 antagonists, the underlying signalling mechanisms appear to be different. In summary, selective non-competitive mGluR antagonists may add by themselves and/or by synergistic effects with chemotherapeutic agents to existing therapies of cancer possibly leading to a useful therapy for drug-insensitive or resistant tumors.

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The growth hormone affects the brain protein synthesis rate in hypophysectomized aged rats

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The purpose of this study was to determine whether the growth hormone (GH) affects the rate of brain protein synthesis in hypophysectomized aged rats. Experiments were conducted on three groups of 24-week-old male rats: group 1 were hypophysectomized to reduce the level of plasma GH, group 2 were hypophysectomized and treated with GH and group 3 were sham-operated controls. The fractional rates of protein synthesis in brains of hypophysectomized rats with GH were significantly greater than that in hypophysectomized rats without GH. In the cerebral cortex and cerebellum, the RNA activity [g protein synthesized/(g RNA.d)] significantly correlated with the fractional rate of protein synthesis ($r > 0.88$, $P < 0.001$). The RNA concentration (mg RNA/g protein) was also related to the fractional rate of protein synthesis in these organs ($r > 0.56$, $P < 0.05$). The results suggest that the treatment of GH to hypophysectomized aged rats are likely to increase the rate of protein synthesis in the brain, and that RNA activity is at least partly related to the fractional rate of brain protein synthesis.

Protein L-isoaspartyl methyltransferase down regulates in epithelial mesenchymal transition of the breast cancer cells associated with integrin

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Epithelial-mesenchymal transition (EMT) occurs during embryonic morphogenesis by which epithelial cells acquire characteristics of mesenchymal cells, appropriate for migration, fibroblast-like properties and reduced cell-cell adhesion. Recent advances support the idea that EMT has a central role in tumor progression. Protein methylation reactions utilize methyl groups derived from dietary donors by the action of methyltransferases. Protein *L*-isoaspartyl *O*-methyltransferase (PIMT, EC 2.1.1.77) is known to repair damaged proteins which have accumulated abnormal aspartyl residues during cell aging. Recent studies show PIMT levels were regulated by cell adhesion in various cancer cell lines involving the integrin $\alpha v\beta 3$ and PI3 K pathway. Also others demonstrated the possibility of cross-talk between E-cadherin and v integrin in breast cancer lines. We examined the relationship between PIMT and EMT associated with integrin v using human breast cancer cells, non-invasive MCF-7, invasive MDA-MB 231 and normal breast epithelial cell MCF10A. During cell detachment the PIMT and E-cadherin levels were rapidly and strongly increased correlated with down regulation of integrin v. We also observed that aggregation occurred in detachment of the cells. But the cell aggregation could be prevented by treatment with adenosine dialdehyde, a PIMT inhibitor. We induced EMT in breast cancer cells by the treatment with TGF and TNF. The expression level of PIMT was negatively regulated by alteration of phosphorylation of extracellular signal-regulated kinase (ERK) and glycogen synthase kinase 3 (GSK3), While EMT makers' level rose up. We revealed to link between EMT and PIMT expression related with ERK and GSK3 signaling pathway in breast cancer cells.

Alternation of immune responsiveness in a rat model of Parkinson's disease

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Previous studies have suggested that central dopaminergic pathways are asymmetrically involved in the modulation of immune response. In order to study the involvement of post lesion neuronal structures in these results, some immunological parameters (total serum protein, lymphocyte and antibody titer) were determined 1 week after right unilateral lesion of the substantia nigra (SN) by means of 6-hydroxydopamine. We showed that the immune response decreased no significantly in right lesioned animals. Based on these findings, we have suggested that asymmetry in brain immunomodulation involves functionally related dopaminergic and cortical networks.

Stimulation of interferon-g production by garlic lectin in the mouse spleen cell through cell surface binding sites shared with concanavalin A

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Several lectins, present in beans and other edible plant products, have been shown to have immunopotentiating and anti-tumor activities. We investigated the immunomodulatory effects of garlic lectin purified from garlic bulbs, and here report the effect of the lectin on interferon-γ (IFN-γ) production in mouse spleen cells. The garlic lectin (0.5–2.0 mg/ml) induced IFN-γ production in the spleen cells in a time course (24–60 h), and the maximal enhancement was observed at 36 h with 0.5 mg/ml of the lectin. The garlic lectin also caused an increase in IFN-γ mRNA level. U0126 and PD98059, inhibitors of extracellular signal-regulated kinase (ERK), inhibited the stimulatory effect of lectin on IFN-γ production. Furthermore, the lectin (0.5 mg/ml, 24 h) increased the

phosphorylation of ERK in the cells. Garlic lectin inhibited the binding of concanavalin A (Con A) to the spleen cell surface in a competitive manner (Ki value = 28 mM). The present findings suggest that garlic lectin binds to the cell surface sites shared with Con A and induces IFN-γ production via activation of ERK in mouse spleen cells.

Production and evaluation of monoclonal antibodies against human epidermal growth factor receptor in Balb/c mice

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Characteristically, the malignant diseases are self sufficiency in growth signals, resulting in avoidance from apoptosis, induction of angiogenesis and metastasis. Thus, one of the most persistent cancer therapy modality appears to be targeting the cancer-specific antigens using monoclonal antibodies (mAbs). Of these biomarkers, human epidermal growth factor receptor (hEGFR) has been shown to be overexpressed in various cancers. Here, we report production of mAbs against hEGFR in Balb/c mice. Six female Balb/c mice (6–8-week-old) were immunized against A431 tumoral cells, which highly express EGFR, in four periods. The most immune mouse was selected for fusion with sp2/0 cells (myeloma cells) in presence of polyethyleneglycol. Supernatant of hybridoma cells were screened for detection of antibody using ELISA. Large scale production of mAbs were obtained in vitro and ascetic fluid. From 280 clones obtained in this work, 27 displayed absorbance >1. Of these, three clones represented absorbance about 1.7 and selected for limiting dilution (LD). The yield of LD was 8 monoclones with absorbance $\sim$2. These results indicate that the produced mAbs against EGFR can be used in diagnosis and treatment of tumors that express membranous EGFR.

Effect of lysine and vitamin B6 supplementation on male subjects with hypertriglyceridemia

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Lipid profile is known to be affected by fatty acid metabolism. Long chain fatty acid oxidation requires the enzyme carnitine palmitoyltransferase, which in turn depend on the availability of carnitine. Carnitine can be obtained from animal sources or synthesized utilizing lysine and vitamin B6. The effect of lysine and vitamin B6 supplementation on male subjects with high level of triglyceride (TG > 150 mg/dl) was studied. Eighty one hypertriglyceridemic subjects were randomly divided into four groups: Placebo, lysine (1 g/day), vitamin B6 (50 mg/day) and lysine plus vitamin B6. Subjects were maintained on the supplement for 12 weeks and plasma glucose and lipid profile were tested at baseline, 6 and 12 weeks.

Lysine supplement did not alter lipid profile, but plasma glucose was reduced at 6 weeks. Vitamin B6 and lysine plus vitamin B6 groups showed a decrease improvement in some components of lipid profile mainly related to total cholesterol, LDL and HDL, but TG and glucose were not affected. Although the subjects were all hypertriglyceridemic, but most had other abnormalities in terms of the other components of the lipid profile or glycemia. These confounding factors may have affected the results and more controlled studies are needed. The mechanism(s) by which lysine and vitamin B6 exerted their effect is not clear, especially that both are known to have several metabolic effects.

Lysine and vitamin B6 supplement were found to exhibit some effect on the glycemic and lipidemic profiles of subjects with high triglyceride levels.

Alterations of intrinsic amounts of proline and hydroxyproline enantiomers in mammalian skin with aging

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D-Amino acids are getting recognized as the novel physiologically active substances and biomarkers in mammals. In mammalian skin, collagen is damaged with aging and UV irradiation, which sometimes results in the racemization of the amino acid residues. Therefore, alterations of intrinsic amounts of proline (Pro) and hydroxyproline (Hyp) enantiomers in the skin, the major amino acid residues of collagen, is expected to be clarified. For the determination of extremely small amounts of Pro and Hyp enantiomers, a fully automated 2D-HPLC method employing a microbore-monolithic ODS column (0.53 mm ID) and a narrow-bore-enantioselective column (prototype Chiralpak QN-2-AX of 1.5 mm ID) has been developed. The amino acids were detected by the fluorescence after derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). Using the present method, intrinsic amounts of D-Pro in the mouse epidermis and dermis were successfully determined. In the epidermis, the amounts of D-Pro in 4-week-old mice were about 10 times higher than those in the mice of 6–45 weeks of age. This remarkable alteration was not observed for D-serine and D-alanine. In contrast, D-Pro in the dermis was gradually increased with aging. Throughout the investigation, D-Hyp was not detected in all the tissues tested. For further works, determination of Pro and Hyp enantiomers in the skin under various conditions such as dry or diseases is currently in progress.The present study was approved by the ethics committee of Shiseido Research Center in accordance with the guideline of the National Institute of Health.

Proteomic analysis of childhood acute lymphoblastic leukemia by two-dimensional gel electrophoresis coupled with MALDI-TOF mass spectrometry

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Acute lymphoblastic leukemia (ALL) is a malignant disease of the blood and the bone marrow and is considered the most common type of childhood cancer. Although the rates of success in treatment of ALL are steadily increasing, further attempts may lead to a more favorable therapeutic outcome. The purpose of this study was to verify a list of protein candidates relevant to the prediction of the clinical behavior and personalized treatment of childhood ALL when performed on plasma from bone marrow (BM) and peripheral blood (PB). Twenty eight plasma samples from BM and PB collected from patients with childhood ALL at diagnosis were analysed. Diagnosis was based on morphology, cytochemical staining, cytogenetic analysis, molecular and immunophenotypic study. As controls, four plasma samples from BM and PB from non-leukemic pediatric patients were studied. Differential proteomic analysis was performed by two-dimensional gel electrophoresis followed by MALDI-TOF mass spectrometry. Fifty proteins were found differentially expressed in ALL BM plasma samples and 60 proteins were found to be expressed in PB plasma as compared to control. By these, 35 proteins were detected in both BM and PB plasma of the ALL pediatric patients. The majority of the identified proteins included suppressor genes, metabolic enzymes, structural proteins and signal transduction mediators. Our study demonstrates the potential of proteomic technology for the identification and confirmation of protein composition in plasma. However, a comprehensive knowledge of the value of these proteins is required in order to elucidate its relevance on childhood ALL screening, diagnosis and treatment outcome.

Neuroscience

Extrasynaptic NR2D-containing NMDARs mediate tPA-promoted excitotoxicity

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Although the molecular bases of its actions remain debated, tissue-type plasminogen activator (tPA) is a paradoxical brain protease, as it favours some learning/memory processes but increases excitotoxic neuronal death. Here we demonstrate that in cultured cortical neurons, tPA selectively promotes extrasynaptic but not synaptic *N*-methyl-D-aspartate receptor (NMDAR)-dependent activation. We show the tPA-mediated signalling and neurotoxicity through the NMDAR is blocked by co-application of a NR2D antagonist (PPDA) or knock-down of neuronal NR2D expression. In addition, activation of synaptic NMDAR prevents further tPA-dependent NMDAR signalling and neurotoxicity. This study demonstrates that the previously described pro-neurotoxic effect of tPA is mediated by extrasynaptic NR2D-containing NMDAR-dependent Erk(½) activation, a deleterious effect prevented by synaptic pre-activation.

Reduction of caspase-3 activity upregulates the glutamate transporter GLT-1 in cultured white matter astrocytes from a rat model of amyotrophic lateral sclerosis (hSOD1^{G93A})

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Amyotrophic lateral sclerosis (ALS) is typically characterized by a dramatic loss of lower motor neurons in spinal cord and brainstem. Nevertheless, upper motor neuron dysfunction is also reported in ALS and frequently related to a deficit of transcallosal connections associated to a reduced volume of the corpus callosum. Besides, impairment of the astroglial glutamate transporter GLT-1 associated with accumulation of extracellular glutamate is demonstrated in ALS and related excitotoxicity is likely to participate in the progression of the disease. At the molecular level, the caspase-3-mediated cleavage of GLT-1 leading to a selective and functional inhibition of the transporter was evidenced in spinal cord homogenates of a transgenic mice model of ALS.

We herein characterised the expression and activity of GLT-1 and caspase-3 in cultured callosal astrocytes isolated from a transgenic rat strain expressing an ALS-related mutated form of human superoxide dismutase 1 (hSOD1^{G93A}). Quantitative RT-PCR and Western-blotting studies revealed that the expression of GLT-1 was higher in the cells prepared from the transgenic animals in comparison to the wild-type rats. However, specific measurements of D-[^{3}H]-aspartate uptake velocity failed to evidence differences in the activity of this transporter. Nevertheless, measures of uptake in the presence of a caspase-3 inhibitor suggested that a reduced activity of this apoptotic enzyme, which is highly detected in callosal astrocytes from hSOD1^{G93A} rats, could exclusively upregulate the GLT-1 activity in cells from transgenic animals.

Together, these findings reinforce the hypothesis of an involvement of caspase-3-mediated impairment of glutamate uptake in the pathogenesis of ALS.

Identification of additional *Escherichia coli* AtoC binding target elements gives new insight to bacterial regulatory networks: molecular modeling of AtoC-DNA binding domain amino acids

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Bacterial environmental adaptation is mediated by networks of cross-talk between two component systems, while signal transduction takes place via phosphor-transfer from sensor histidine kinases to aspartic residues of response regulators, the signal output carriers. Transcriptome and phenotypic analyses indicated the involvement of the AtoSC two component system of *E. coli* in various cellular activities, apart from the activation of the *atoDAEB* operon expression, upon acetoacetate induction. The activated response regulator AtoC is reported to act as transcriptional activator and its *cis*-regulatory binding site was identified as a palindromic repeat within the *atoDAEB* promoter.

The nature of the bonds formed between the AtoC amino acids and the specific DNA residues was investigated with molecular modeling. Moreover, in an attempt to define novel AtoC binding elements which would indicate an AtoC-related cross-regulatory function, we employed an *ab initio* motif detection approach, coupled with gene ontology analysis. The genome-wide computational screening indicated several putative matching sites within the *E. coli* genome. Chromatin Immunoprecipitation assays were carried out to assess the *in vivo* binding of AtoC to the predicted sites. This process verified twenty-one additional AtoC binding sites, albeit their sequence divergence, located not only within intergenic, but also within gene-encoding regions.

The elucidation of the molecular conditions governing the AtoC binding to these sites in vitro and in vivo, its dimerization and putative participation of other factor(s) gives an insight to a possible AtoC-related regulatory network which may contribute to the pleiotropic effects of AtoC.

In vitro- and in vivo-models of human neuroendocrine tumors: three novel transplantable cell lines from metastatic midgut carcinoid

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Only a few cell lines from human neuroendocrine tumors (NET) have been established so far, among them our earlier KRJ-I cell line from a human ileal carcinoid. The reason for the poor success in establishing carcinoid cell lines is due to the small amount of tissue available and the low mitotic activity in primary cultures. We have successfully established three continuously growing cell lines from tissue obtained from a metastatic human carcinoid of the terminal ileum (midgut carcinoid): P-STS was derived from the primary tumor, L-STS from a lymph node metastasis and H-STS from a hepatic metastasis. Immunocytochemical analysis for NET markers proved the maintenance of pancytokeratin, cytokeratins 7, 8, 18, 19, serotonin (5-HT), NSE, CD56, protein gene product 9.5 (PGP9.5), calcitonin, synaptophysin, and gastrin releasing factor. The presence of electron-dense neuroendocrine granules was demonstrated by electron microscopy and quantified by immunogold toning of 5-HT. The three cell lines were tumorigenous in SCID mice. Cytogenetic analyses revealed clonal tetraploidy, inversion and deletion in chromosome 18q, and non-clonal numerical and structural aberrations. Array CGH did not show notable imbalances. Mutation screening of P-STS excluded a MEN1-gene-associated genetic predisposition. The novel carcinoid cell lines may serve as valuable models for the elucidation of proliferative and secretory mechanisms as well as for the study of new therapeutic agents.

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Endomorphin 2-like immunoreactive peptide is generated extracellularly in rat isolated L-4,5 dorsal root ganglia and *in vivo* in the spinal cord dorsal horn from Tyr-Pro precursor by a membrane-bound DPP-IV-like enzyme in a depolarization-stimulated manner

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Background: The potent and mu-opioid receptor selective agonist mammalian brain tetrapeptides, endomorphin-1 and -2 (E2) were discovered in 1997. The gene(s) encoding for their precursor(s) is/are still unknown. We have found some evidence for a potential de novo biosynthetic route starting from Tyr-Pro precursor. Presently, we measured the generation of immunoreactive E2 in adult rat isolated L4,5 dorsal root ganglia and also in vivo in the spinal cord in carrageenan-induced hyperalgesia.

Results: In rat isolated dorsal root ganglia the combination of presumed biosynthetic precursor of E2, Tyr-Pro with the prolyl peptidase DPP-IV enzyme inhibitor Ile-Pro-Ile generated 1.60 $\pm$ 0.37 pg/mg Wet Tissue Weight_30 min immunoreactive E2 in the bathing fluid ($n = 4$) with an eightfold increase upon depolarization whereas the tissue content was low (0.50 $\pm$ 0.08 pg/mg_WTW). Substance P, determined by ELISA, was found almost exclusively within the tissues. *In vivo* intrathecally injected 30 nmol Ile-Pro-Ile was antihyperalgesic in carrageenan-induced hyperalgesia (hindpaw, Randall-Selitto). The antihyperalgesic effect was antagonized by 1 mg/kg naloxone *sc* and also by intrathecal antiserum to E2 (see poster by Király et al.).

Conclusions: In isolated dorsal root ganglia E2-IR was generated extracellularly by a membrane-bound DPP-IV-like enzyme, which was depolarization-sensitive in "synthase" functional mode. The shift to "synthase" was induced by the DPP-IV hydrolase inhibitor Ile-Pro-Ile. In vivo "C fiber-mediated wind-up" induced by carrageenan created conditions favoring endomorphin generation in the spinal cord dorsal horn when the enzyme was shifted to „synthase" function. This assumption may open a new approach to pain alleviation in some chronic pain states.

Hippocampal protein expression levels linked to spatial memory formation in laboratory and wild-caught mouse strains

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The cognitive dependency on strains and tasks is well-documented, no systematic studies on wild-caught mouse (WCM) strains done so far. Moreover, no information is available on spatial learning related hippocampal signaling protein (hSPs) expression levels in WCM. We aim to study spatial memory in Morris water Maze (MWM), Barnes Maze (BM) and Multiple T-Maze (MTM) with WCM strains, *Apodemus sylvaticus* (AS), *Mus musculus domesticus* (MMD), *Mus musculus musculus* (MMM) and laboratory mouse strains, C57BL/6 J, CD1 and PWD/PhJ. The strains chosen for identification of hSPs based on performance in MWM.

Male, 20 per group, 12–14 weeks old, were used in the experiments. Equal number of animals used as yoked controls, were not trained in the MWM.

The learning was determination by time(s) spent in the target quadrant, visits to the target hole and correct decisions at time points 5 (D5) and 12 days (D12) in MWM, BM and MTM respectively. Individual hippocampi were dissected and sample preparation was conducted, which followed by analysis of hSPs with immunoblotting.

Strain and task-dependent performance was observed across the strains. The latency to reach the platform as well as path length decreased significantly in AS compared to other strains. Moreover, expression levels of hSPs correlate with the performance in MWM.

We learn from this study that a series of hSPs are associated with spatial memory and that different hSPs are linked to spatial memory in different strains that show different outcome in the MWM. Even correlation patterns in the individual hSPs differed between mouse strains.

Glycine receptors contribute to hypnosis

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Glycine is a major inhibitory neurotransmitter in the CNS, and its receptors (GlyRs) are well known for their effects in the spinal cord and lower brainstem. Accumulating evidence indicates that GlyRs are also present in higher regions of the brain, including areas thought to be involved in consciousness. It is well documented that several general anesthetics, including propofol and ethanol potentiate brain GlyR function, yet the behavioral role of brain GlyRs has not been well explored. Here we used the loss of righting reflex (LORR) as a marker of hypnotic state, because the concentrations of anesthetics that are necessary to produce hypnotic state in humans are similar to those needed to induce LORR in rodents. We found that systemically administered propofol, ethanol and ketamine dose-dependently induced (LORR) in rats. Furthermore, systemically administered strychnine dose-dependently reduced the percentage of rats exhibiting LORR induced by propofol and ethanol, increased the onset time, and decreased the duration of LORR. Strychnine had no effect, however, on the LORR induced by ketamine. Moreover, propofol markedly increased the currents elicited by glycine and GABA of hypothalamic neurons. Conversely, strychnine and GABAzine both profoundly attenuated the current induced by propofol. Given that hypnosis is due to neuronal depression in upper brain areas, we therefore conclude that brain GlyRs contribute at least in part to the hypnosis induced by propofol and ethanol.

Modulation of mGluR5 protein expression by association of CAL

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In this study, we investigated the association of metabotropic glutamate receptor subtype-5 (mGluR5) with cystic fibrosis transmembrane conductance regulator-associated ligand (CAL). By using GST pull-down techniques, we found that the mGluR5 directly interacted with CAL via carboxyl-terminus of receptor bound to the PDZ domain of CAL, and the C-terminal last four amino acids (S–S–S–L) of the receptor were the essential determinants for this interaction. Consistent with the results of the fragment interactions in vitro, full-length mGluR5 also associated with intact CAL in vivo, as determined by coimmunoprecipitation experiments and an *immunofluorescence assay*. Functionally, co-expression with mGluR5, CAL profoundly enhanced the expression of mGluR5 in protein but not in mRNA levels, possibly by blocking ubiquitination-dependent receptor degradation. These findings revealed the physiological regulation of mGluR5 protein expression via the interaction with CAL, and suggested a molecular mechanism by which the expression of mGluR5 protein could be regulated at the post-translational level via PDZ scaffold protein CAL.

Glutamine synthetase activity, expression and distribution in the brain and the spinal cord of an animal model of amyotrophic lateral sclerosis ($hSOD1^{G93A}$ rats)

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Excitotoxicity induced by glutamate accumulation is directly implicated in the progression of amyotrophic lateral sclerosis (ALS). Indeed the progressive and selective motor neuron degeneration has been frequently correlated with a disruption of glutamate homeostasis. Consistently, dysfunction of glial glutamate transport activity is evidenced in transgenic rodents expressing an ALS-related mutated form of human superoxide dismutase 1 ($hSOD1^{93A}$). Once taken up by astrocytes, glutamate is metabolized by glutamine synthetase (GS), a key enzyme in the glutamate-glutamine cycle between neurons and glia.

We recently evidenced distinct glutamate handling properties in white and grey matter astrocytes and we are currently considering these differences in the context of ALS. The goal of the present study is to examine potential modification of the expression and/or activity of GS in white and grey matter structures of the brain and the spinal cord of $hSOD1^{G93A}$ rats at end-stage compared to wild-type animals at the same age. The measure of GS activity in samples from selected brain and spinal structures by spectrophotometry revealed the lack of significant changes during the progression of the disease. Considering the massive gliosis observed in the brainstem and the spinal cord of these animals, the absence of GS regulation is certainly unexpected and additional studies by Western blotting and immunohistochemistry are in progress in order to examine putative changes in the expression and cellular distribution of the enzyme in these animals.

Protein isoaspartyl methylation enhanced neuronal differentiation of bFGF treated PC12 cells

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Protein L-isoaspartyl methyltransferase (PIMT, EC 2.1.1.77) is a cytosolic enzyme that methylates the side chain carboxyl groups of racemized D-aspartyl or L-isoaspartyl residues in proteinaceous substrates with *S*-adenosylmethionine (AdoMet) as a methyl donor. Expression level of PIMT is high in the brain but the functions of PIMT are poorly understood. We have analyzed the importance of protein L-isoaspartyl methylation by *S*-adenosylmethionine-dependent methyltransferases for neuronal differentiation of rat pheochromocytoma cells (PC12). We showed that treatment of cells with methyltransferase inhibitor adenosine dialdehyde (AdOx) and PIMT siRNA decreased the level of neuronal class III tubulin (Tuj1). We attempted to elucidate the role of PIMT on growth factor-induced neuronal differentiation in PC12 cells. In response to basic fibroblast growth factor (bFGF), PC12 cells differentiate into sympathetic-like neurons. These differentiated neuronal cells elevated levels of the neuronal differentiation markers, neurofilament H, M (NF-H, M) and Tuj1. Moreover, we have shown that the mitogen-activated protein kinase (ERK1/2) is persistently activated during neuronal differentiation, and conversely, ERK activation is inhibited while treatment of the cells with PIMT siRNA. Stimulation by bFGF resulted in phosphorylation of Akt, GSK3 and an increased level of PIMT. Taken together, these findings suggest that methylation reactions might influence the neuronal differentiation of PC12 cells and bFGF induces elevated level of PIMT in PC12 cells through Akt-GSK3 activation, which leads to ERK activation and neuronal differentiation.

Inhibitory effects of protein L-isoaspartyl *O*-methyltransferase in amyloid β aggregation in vitro

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Many age-related degenerative diseases, including Alzheimer's, Huntington's and Parkinson's disease, are associated with accumulation of amyloid fibrils. Fibrillar aggregates of the β-amyloid peptide (Aβ) are major constituents of the senile plaques found in the brains of Alzheimer's disease (AD) patients and have been related to AD neurotoxicity. It has been shown that spontaneous isomerization or racemization of aspartyl residues of Aβ peptides led to conformational changes in secondary structures and to increase of aggregative ability. Protein L-isoaspartyl *O*-methyltransferase (PIMT, EC 2.1.1.77) converts L-isoaspartyl/D-aspartyl residues in damaged proteins to normal L-aspartyl residues. We demonstrated that PIMT had inhibitory effects on Aβ aggregation in vitro via isoaspartyl methylation. Thioflavin-T (Th-T) binding assay after aging in vitro (37, pH 7.4 in PBS) showed that PIMT had tendency of inhibition against Aβ fibrillization. In western blotting, high molecular Aβ aggregates (>200 kDa) are occurred in only Aβ incubation but those are reduced in incubation with PIMT. We determined the level of the β-sheet transition in secondary structure by measuring circular dichroism (CD). Aggregation of Aβ pentamers is observed by using transmission electron microscopy (TEM). We also investigated the role of PIMT on the prevention of Aβ fibrillization enhanced by metal ions ($Fe3^+$, $Cu2^+$, and Zn 2^+).

Mechanisms of glycine release from spinal and hippocampal glycinergic nerve terminals in response to depolarizing stimuli

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Glycine release from glycinergic nerve terminals has been rarely studied. We investigated the release of the amino acid from mouse spinal cord and hippocampal synaptosomes prelabelled with [^{3}H]glycine in the presence of the GLYT1 transporter blocker NFPS, to achieve selective labelling of glycinergic terminals through GLYT2. Synaptosomes were depolarized in superfusion with different concentrations of KCl, 4-aminopyridine (4-AP) or veratridine. The [^{3}H]glycine overflow evoked by identical depolarizing stimuli was significantly higher in spinal cord than in hippocampus. KCl ($\leq$15 mM), 4-AP, and veratridine ($\leq$0.3 μM) provoked [3H]glycine release by external Ca^{2+}-dependent exocytosis in both regions. In spinal cord, the overflows evoked by higher concentrations of KCl or veratridine involved external Ca^{2+}-independent mechanisms; surprisingly, in the hippocampus the 50 mM KCl-evoked [^{3}H]glycine overflow was almost totally dependent on external Ca^{2+}; moreover the external Ca^{2+}-dependent fraction of the 10 μM veratridine-induced overflow in the hippocampus was higher than in spinal cord. Part of the veratridine-evoked overflow occurred by GLYT2 reversal; in contrast, the external Ca2+-independent overflow provoked by 50 mM K^+ in spinal cord was transporter-independent. The external Ca^{2+}-independent, transporter-independent components of the overflow evoked by 50 mM KCl or 10 μM veratridine were largely sensitive to Bafilomycin A_1 and prevented by blocking the mitochondrial Na^+/Ca^{2+} exchanger with CGP 37157, indicating the involvement of exocytosis triggered by intraterminal mitochondrial Ca^{2+}. The multiplicity of glycine actions and the primary roles of glycinergic neurotransmission in several physiological and pathological processes justify a better understanding of glycine release and its modulation.

Characterization of GABAA receptor alpha3 subunit interaction with gephyrin

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Synaptic GABAA receptors are frequently associated with submembranous aggregates of gephyrin. A gephyrin interaction site within the GABAA receptor alpha2 subunit intracellular loop has recently been characterized by using hippocampal cultures and biochemical methods. The presence of a linker protein between the GABAA receptor and gephyrin could be ruled out by overlay assays, where only the two interaction partners were present in the biochemical experiment. Here we report that similar overlay assays and the yeast two-hybrid system also reveal a strong interaction of the GABAA receptor alpha3 subunit with gephyrin. Only a minor population of GABAA receptors contains the alpha3 subunit, mostly in monoaminergic neurons and in the nucleus reticularis thalami (nRT). In GABAA receptor alpha3 subunit

knockout mice there is no up-regulation of other alpha subunits in nRT neurons, while the clustering of the gamma2 subunit is disrupted. Gephyrin forms large aggregates in the nRT neurons of these mice, that in most cases are not associated with synaptic sites. These results are again an example of a dominant role of an alpha subunit in synaptic GABAA receptor and gephyrin clustering. Here we determine the interaction site between the alpha3 subunit and gephyrin.

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Spider toxins as tools to elucidate insect receptors

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Precise identification of binding site and mode of action of several spider toxins on insect receptors and their potential in the development of new bioinsecticides still remains an important underdeveloped area. Palutoxin IT1 (Palu IT1) an insecticidal toxin isolated from the venom of the spider *Paracoelotes luctuosus* (Araneae: Amaurobiidae) can be used as an important tool to elucidate insect receptors in crop pest larvae such as *Spodoptera frugiperda*. Previously Palu IT1 insecticidal effect was determined in *Spodoptera litura*. In this work, a synthetic analogue of the native and FITC fluorescent label Palu IT1 were used to determined LD_{50} value by an in vitro microinjection assay in third instar larvaes pronotum. As first approximation, a window bioassay was done, using a high-low based dose test. Once concentrations to be used were determined, a completely random design test was stablished, including seven treatments and negative control, with three replicates of 15 larvaes units each one. Statistical analysis using Probit (POLO 1.0 program) was performed to confirm in vitro results. The first toxin effects were clearly observed after half of an hour, symptons as paralysis, tissue necrosis and death were observed in the larvaes after 24 h. Death larvaes number and the LD_{50} were determinated 6.3 $\pm$ 0.2 μg/g larvae. The toxicity of Palu IT1 towards *S. frugiperda* was similar to the reported for *S. litura* (9.5 $\pm$ 0.3 μg/g larvae). FITC-flourescent label toxin was an important tool to elucidate receptors in the larvae tissue.

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Nutrition

Chemopreventive properties of dietary Bowman–Birk inhibitors from legumes

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The chemopreventive properties of naturally-occurring protease inhibitors (PI) of the Bowman–Birk class from legume seeds, and their possible role in the prevention and treatment of cancer within the gastrointestinal tract (GIT), have been investigated. Bowman–Birk inhibitors (BBI) are highly resistant to the extreme conditions of the GIT and significant amounts reach the large intestine in an intact form. The protease inhibitory activities are intrinsically linked to the chemopreventive properties of BBI proteins and are largely unaffected by intestinal microbiota. BBI are significant, therefore as bioactive compounds in the large intestine. Soybean BBI have been investigated extensively for potential anti-carcinogenic effects, both in purified form and as a protein extract enriched in BBI (BBIC). These have been shown to be effective at preventing or suppressing colorectal carcinogenesis in a range of in vitro and in vivo animal model systems. In addition to cancer chemopreventive properties, soybean BBI have shown anti-inflammatory activity in animals exposed to carcinogenic agents, an observation that has been extended to patients with ulcerative colitis. All these findings imply a positive contribution of naturally-occurring BBI proteins to the nutritional value of dietary proteins from legumes and suggest that soybean BBI and related proteins may be exploited in cancer prevention programmes and for further medical applications within the GIT.

Accumulation of D-tryptophan in the insides of rat due to administration of D-tryptophan containing fodders

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Several studies have been published about the effect of D-amino acids on the living organisms. Among them, however, no study dealing with D-tryptophan was found. As in the passed years we elaborated a method for the determination of D-Trp, as a continuation of this experiment we tested the effect of D-Trp entering the organism along with the fodder on rats. The effect of one single high dose of D-Trp, as well as the accumulation in organs due to a longer continuous administration was examined. After the feeding phase D-Trp content of the liver, kidney and spleen of the rats, as well as in the case of the single feedings that of the chymus was determined. A single high dose of D-Trp did not result in detectable D-Trp content in the organs, whereas in case of the longer treatment D-Trp was found in the kidney and the spleen but not in the liver. The reason for this may be that there is in the liver a D-amino acid oxidase enzyme system that can desaminate the D-amino acids into α-keto acids, which are able to convert into L-amino acids.

Effect of fortification with amino acids and proteins on the quality and volatiles of corn flour extrudates

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The amino acids and vegetable proteins have been recommended as safe flavouring precursors of thermal process flavouring. Therefore the present work was designed toexamine the effect of

such compounds on the overall qualities of corn flavour extrudates. Amino acids(proline and cysteine), vegetable proteins(soybean and gluten) and sucrose were added to corn flour pre-extrusion process. A sample free from any additives was considered as control. The sensory evaluation for each sample was carried out, the expansion ratio and protein content were determined, the results showed a significant increase in all sensory attributes comparing with the control sample. Samples containing proline or cysteine showed high quality attributes whereas addition of soybean protein or gluten resulted in significant decrease in appearance and texture attributes compared with the sample containing sucrose only. An agreement between the sensory results and expansion ratio for all samples was observed for the protein content. On the other hand, theGC andGC–MS analysis confirmed the aroma quality of each sample.

Does leucine of high protein diets play a role in the regulation of energy balance in mice?

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High protein (HP) diets can lead to reduced body weight, increased fat oxidation, and a better preservation of lean body mass as compared to low protein diets. Furthermore, a HP intake can stronger promote satiety and energy expenditure in comparison to carbohydrates or fat. It was suggested that branched chain amino acids of dietary proteins, e.g. leucine, could act as nutrient signals and might be involved in the regulation of specific gene expressions involved in modifications of food energy efficiency and energy balance.

To clarify the effect of HP diets and leucine on energy balance male C57BL/6 mice were fed for 20 weeks with experimental diets containing either 10% (control) or 50% whey protein (WP). A third group was exposed to a 10%WP diet supplemented with L-leucine (WP + L) as contained in the 50% WP diet.

HP diet fed mice had a significant lower body weight, food intake, and body fat mass (measured by quantitative magnetic resonance) as compared to other groups. Food energy efficiency (g/MJ) was 2.14 ± 0.08, 1.11 ± 0.08 and 1.57 ± 0.11 for control, WP and WP + L, respectively. Resting and total energy expenditure (kJ/g/d) was higher of HP and WP + L fed mice compared to controls. Leucine supplementation resulted in effects intermediate to those of the HP group and controls supporting a significant role in energy balance regulation.

The role of protein in body weight regulation was confirmed. However, further research is required to clarify the biochemical mechanisms. The results may have an impact in prevention and treatment of human obesity.

Waste of fish-processing industry as source of protein substances

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Marine organisms of the Northwest part of Russia are unique renewed raw materials for production of various natural substances. A protein-containing waste from commercial catch and industrial processing of marine hydrobionts make up a considerable part of these raw materials: sub-standard muscle of the Icelandic scallop; sub-standard shrimps; shells of King crabs; not trade kinds of crabs, shellfishes, etc.; the waste formed at industrial processing of fish. A protein-containing waste reach to 60% on mass of all volume of catch. To cheap protein-containing raw material it is necessary to refer also invaluable species of fishes: polar cod, blue whiting, marine cock (grey Trigla), slopes, etc.

Rational exploitation of marine raw materials assumes its processing without waste. One of the basic directions of a re-use of not used protein-containing wastes consists in production of enzymatic protein hydrolysates. These hydrolysates can be applied as a protein component of microbialogic media, feed components to fishes, poultry and agricultural animals, nutrient solutions for postoperative patients, etc.

The internals of hydrobionts now are waste of industrial processing and use very poorly. Possibility of proteinase production from these internals is shown too, that matters for the decision of a problem of rational processing of marine resources. The complex enzyme preparation from the hepatopancreas of the acclimatised King crab already has found application in production of enzymatic protein hydrolysates.

Economic feasibility of the offered complex processing of a protein-containing waste at Murmansk enterprises is shown.

Cellular and animal responses to indispensable amino acid deficiency are mediated through the integrated stress response pathway

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The integrated stress response pathway (ISR) a defense mechanism cells employ when under stress (e.g. amino acid deprivation), causes suppression of global protein synthesis along with the paradoxical increased expression of a host of proteins that are useful in combating various stresses. Microarray analyses on HepG2/C3A cells cultured in cysteine or leucine deficient medium showed induction of genes involved in amino acid uptake, aminoacyl-tRNA synthesis and transcriptional regulation, while genes involved in cell cycle/division and nucleotide synthesis were down-regulated. Animal studies were conducted to investigate if observations from the microarray experiments hold in an animal model. Rats fed a sulfur amino acid-deficient diet for 7 days grew at a much lower growth rate compared to control for the first 4 days and increased their growth rate almost to that of control for the remainder of the experimental period, indicating adaptation to stress. In those rats, phosphorylation of eIF2α was observed (exhibiting sustained ISR induction) as well as induction of protein levels of ATF4, ATF3, ASNS, SLC7A11, and CARS, offering further evidence for the induction of the ISR and adaptation to amino acid deficiency. Expression levels of TRIB3 and GADD34 were not increased indicating that apoptotic pathways were not induced. Taken together, these data demonstrate that rats consuming a sulfur amino acid-deficient diet have a prolonged activation of the ISR pathway which offers evidence in support of ISR as an adaptation mechanism for mammalian cells to deal with prolonged stress and, in the case of this study, improved growth on an amino acid-deficient diet.

Biochemical aspects of homoserine kinase in quality protein maize

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The main deficiency of cereal seeds is the low content of some essential amino acids such as threonine, resulting in low grain nutritional quality. In plants and microorganism aspartate serves as a precursor for the synthesis of lysine, methionine, threonine and isoleucine. The enzyme homoserine kinase (HK) produces *O*-phospho-homoserine (OPH) which is used for methionine synthesis and threonine synthesis. In this work, a wild type maize line (L161) and two Quality Protein Maize (QPM) lines (L161o and L161q) were used to isolate HK. Seeds were harvested at 14, 20 and 24 days after pollination (DAP). The highest level of HK activity was observed at 14 DAP in L161n and L161o lines. However, at 20 and 24 DAP a reduction in enzyme activity was observed. The L161q line exhibited the same HK activity pattern during endosperm development. The addition of isoleucine to the assay mixture induced a strong inhibition of HK activity from 14, 20 and 24 DAP in L161n. HK activity was significantly inhibited by threonine in L161o and L161q lines at 24 DAP. SAM was also able to inhibit HK activity in L161q at 20 DAP. However, the addition of lysine and methionine to HK assay did not produce any inhibitory effect. These results are an important part of a broader picture to understand the mechanisms responsible for increased protein quality in the QPM varieties.

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Studies on natural ^{15}N and ^{13}C isotopic abundances (IA) in amino acids (AA) of humans, implications of dietary and physiological factors

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Stable ^{15}N and ^{13}C IA (δ-values) of human tissue proteins can be influenced by dietary protein and by physiological factors. However, bulk measurements do not allow for more differentiated investigations in contrast to studies at the individual AA level. We are using the derivatization of AA into their *N*-pivaloyl-*i*-propyl esters to investigate the δ-values of up to 15 proteinogenic AA by GC/C/IRMS at the natural abundance level. Characteristic natural patterns of ^{15}N and ^{13}C IA were measured in individual AA of nutritional protein sources, human plasma, and hair. The isotopic patterns differed between sample sources and the results confirmed considerable differences of IA between individual AA. The differences between the highest $\delta^{15}N$ values of proline and the lowest values of threonine were about 22‰. The highest $\delta^{13}C$ values of glycine and the lowest of lysine differed by up to 31‰. The patterns of hair AA-specific $\delta^{15}N$ and $\delta^{13}C$ values were comparable with those of plasma protein AA. Leucine could be a marker for diet-related and physiological influences on IA. The ^{15}N and ^{13}C IA of leucine in hair positively correlated with habitual animal protein intake ($R = 0.708$ and 0.512, respectively). Plotting the ^{15}N against ^{13}C IA revealed a clear distinction between indispensability and dispensability of AA nitrogen or carbon. Further, it was shown that a 4-week period of altered meat intake is too short clearly to be reflected in AA-specific IA as expected. Possible reasons are discussed. Our data indicate that AA-specific IA may characterize physiological conditions in more detail than bulk values. However, improvements in methods of AA-specific stable isotope analysis and further investigations on the relationships between AA metabolism and δ-values are required.

Dietary γ-aminobutyric acid affects the brain protein synthesis rate in ovariectomized female rats

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The purpose of this study was to determine whether the γ-aminobutyric acid (GABA) affects the rate of brain protein synthesis in ovariectomized female rats. Experiments were done on two groups of 24-week-old ovariectomized female rats given the 0 or 0.5% GABA added to the 20% casein diet. The concentrations of plasma GH increased significantly with the 20% casein + 0.5% GABA compared with the 20% casein diet alone. In the brain regions, GABA treatment to the basal diet elevated significantly the fractional and absolute rates of protein synthesis. In brain regions, the RNA activity [g protein synthesized/(g RNA.d)] significantly correlated with the fractional rate of protein synthesis. The RNA concentration (mg RNA/g protein) was not related to the fractional rate of protein synthesis. The results suggest that the treatment of GABA to ovariectomized female rats are likely to increase the concentrations of plasma GH and the rate of protein synthesis in the brain, and that RNA activity is at least partly related to the fractional rate of brain protein synthesis.

Bioavailability of tryptophan and methionin in breakfast cereals

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Breakfast is known to be the most important meal of the day and breakfast cereals are often suggested as ideal breakfast choice, since they provide sufficient and balanced amounts of nutrients and energy. The content of proteins and essential amino-acids often serves as an important indicator of their nutritional quality. Therefore, the goal of this work was to assess protein content and bioavailability of tryptophan and methionin in the main classes of breakfast cereals in the market.

Available tryptophan and methionin contents have been investigated in proteins of dinkel pops, barley, rye, corn, millet, oat and rice flakes. Protein content was determined using the Kjledahl method, available tryptophan was determined by the Bates modified method after alkaline hydrolysis and methionin by the McCarthy and Sullivan method after papain hydrolysis of samples. Available tryptophan and

methionin contents were calculated according to the linear regression equation.

Depending on the kind of investigated sample of breakfast cereals, the protein content (mean value of the three series), expressed on dry matter, ranged from 7.11/100 g (rye flakes) to 14.26/100 g (millet flakes). The content of available tryptophan of various cereals ranged from 61.1 mg/100 g (corn flakes) to 116.8 mg/100 g (barley flakes). Methionin content varied from 166.83 mg/100 g (millet flakes) to 422.71 mg/100 g (barley flakes). Taking into consideration the tryptophan and methionin content in proteins of breakfast cereals, the lowest content of both amino acids was found in millet flake proteins (0.49 and 1.17%, respectively), and highest in rye flake proteins (1.07 and 4.32%, respectively).

Peptides

Synthesis and characterization of new radiolabeled CCK8 analogues for targeting CCK2 receptor expressing tumors

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Radiolabeled CCK8 peptide analogues can be used for peptide receptor radionuclide therapy (PRRT) of tumors expressing CCK2/gastrin receptors. We have previously shown that sCCK8 (Asp-Tyr(OSO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) has better characteristics for PRRT than gastrin analogues, partly due to its pan-CCK-R binding properties. Unlike gastrin analogues, sCCK8 shows low kidney uptake. However, sCCK8 contains an easily hydrolyzable sulfated tyrosine residue and two methionine residues which are prone to oxidation. Therefore, we aim to develop new stabilized sCCK8 analogues that are resistant to hydrolysis and oxidation. Therefore, the methionine residues were replaced by norleucine (Nle) or homopropargylglycine (HPG). The Tyr(OSO_3H) moiety was replaced by a robust isosteric sulfonate, Phe(p-CH_2SO_3H). The phenylalanine analogue Phe(p-CH_2SO_3H) of L-tyrosine was synthesized by modification of known synthetic routes. This building block was incorporated in the peptide under normal coupling conditions in solid-phase peptide synthesis without additional side chain protection. Three peptides were synthesized: sCCK8[Phe^2(p-CH_2SO_3H),$Met^{3,6}$], sCCK8[Phe^2(p-CH_2SO_3H),$Nle^{3,6}$] and sCCK8[Phe^2(p-CH_2SO_3H), $HPG^{3,6}$]. All peptides were N-terminally chelated with the macrocyclic chelator DOTA to allow radiolabeling with In-111.

HPLC analysis showed that peptides with Nle or HPG indeed were resistant to oxidation. *In vitro* binding assays on CCK-R expressing AR42 J cells revealed that all peptides showed specific binding and receptor-mediated internalization. Binding affinity values (IC_{50}) of all peptides were lower than 9.5 nM. In vivo studies in tumor-bearing mice showed tumor uptake was highest for sCCK8 and sCCK8[Phe^2 (p-CH_2SO_3H),$Nle^{3,6}$] (4.78 ± 0.64 and 4.54 ± 1.15%ID/g, respectively, 2 h p.i.).

Currently, the dimer of sCCK8[Phe^2(p-CH_2SO_3H),$Nle^{3,6}$] is synthesized and characterized.

Effect of proline substitution on antimicrobial and hemolytic activities of pin 2 analogue peptides

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Pandinin 2 an antimicrobial peptide had been identified and characterized from the venom of the African scorpion *Pandinus imperator*. This α-helical polycationic peptide belongs to the group of antibacterial peptides previously described from several scorpion venoms, frog's skin or the insect's hemolymph. Pandinin 2 had demonstrated high antimicrobial activity against a range of Gram-positive bacteria (2.4–5.2 μM), less active against gram-negative bacteria (2.4–38.2 μM) and inhibited the growth of the yeast *C. albicans.* Its strong hemolytic activity (11.1–44.5 μM) against human, guinea pig and sheep erythrocytes may restrict its medical application. Circular dichroism studies and a high-resolution structure of pandinin 2 determined by NMR showed that the peptide is essentially composed of a single α-helix with mostly a hydrophobic N-terminal sequence and a Pro aminoacids residue at the middle of its primary structure. Four synthetic analogues of Pandinin 2 in which Pro14 was substituted by Val, Gly-Val, Val-Gly, Gly-Val-Gly, were chemically synthesized and antimicrobial and hemolytic assays were done. For antimicrobial experiments, the inhibition agar diffusion assay and liquid media were compared against *Staphylococcus aureus* ATCC 25923 and other bacteria. Pin2 [P14GVG] analogue had highest antimicrobial activity of all in agar diffusion assays without any hemolytic activity. However, in liquid medium analogue Pin2 [P14 V] showed the strongest antimicrobial activity with the same hemolytic activity as Pin 2.

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A gene engineering method for preparing bioactive peptides

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Gene engineering is an efficient method for preparing bioactive peptides. Many naturally occurring peptides have been proved to possess various biological activities and pharmacological activities, such as osteoblastic activity, antitumor activity, analgesic activity, growth promoting activity, and antimicrobial properties. This presentation outlined the specific methods for producing the target bioactive peptides with gene engineering instead of extraction from rare natural material. Primers were designed and synthesized for cloning the gene encoding the target peptide using the preferred codons of *E. coli.* The gene fragments obtained were cloned into the pED plasmid which was reconstructed by inserting a modified gene fragment encoding the C-terminal peptide fragment of L-asparaginase (L-ansB-C) into the two designed restriction endonucleases sites of pET28a. The recombinant expression vector was then expressed in *E. coli* BL21. The sequences of

the recombinant DNAs were determined and verified to be identical to the originally designed for encoding the target bioactive peptides or their analogues. SDS-PAGE analysis was arranged, and showed that expressions of the cloned genes resulted in expected fusion proteins hence protein bands appeared at the right places. These results indicate that cloning of genes encoding the target peptides was successful, as well as constructions of its expression vector and fusion expression system. Several such designed peptides had been removed from the fusion proteins, isolated, purified, and proven to possess some biological activities and pharmacological activities. Further studies are needed to investigate the methods and conditions for isolating and purifying other target peptides from these fusion proteins and evaluating their bioactive properties.

Production of monoclonal antibodies against Ts87 antigen of *Trichinella spiralis* and identification of antigenic peptide from peptide phage display libraries

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Trichinellosis is a global zoonosis mainly caused by *Trichinella spiralis*(*T. spiralis*). We have previously reported that a novel *Ts87* gene from the cDNA library of adult *T. spiralis* was cloned and the recombinant *Ts87* induced a partial protective immunity in mice. In the present study, monoclonal antibodies against Ts87 antigen of *T. spiralis* were obtained by the classical hybridoma technique. Among 1,000 hybridomas derived from the fusion, three monoclonal antibodies (mAbs 2A2, 5A3, 6G12) secreted by hybridomas gave nice results. They all recognized not only the Ts87 recombinant protein but also the native protein from adult or larval and larva tissue setions. A phage peptide library was screened with monoclonal antibody 5A3 to detect the corresponding antigen and simulative mimetopes. The result showed that one of the positive phage clones M7 had homologous peptide sequences with Ts87 recombinant protein. Peptide displayed by M7 phage clone is the corresponding antigen mimetope. Peptide of other positive phage clones may be the simulative mimetope of Ts87 antigen. They can be used as the potential epitope candidates for the vaccine against *T. spiralis* in the future study.

A coarse-grained model for the dynamics of the cell-penetrating peptide, Pep-1, in membrane model systems

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Cell-penetrating peptides (CPP) have been extensively investigated as an alternative means for the delivery of therapeutic agents which display poor cellular uptake and low bioavailability. A primary reason for this is the efficient delivery of the agent by the vector, with both molecules maintaining functional activity once inside the cell. The translocation mechanism through which CPPs cross the biological membrane has been the subject of much debate. It is generally accepted that translocation of the CPP, either alone or in complex with cargo molecule, occurs in a receptor- and energy-independent manner via either the endosomal or non-endosomal pathways. This however, is dependent on the CPP, cargo and cell type. The amphipathic peptide carrier, Pep-1, has been reported to deliver a variety of peptides and proteins into several cell lines without prior chemical covalent coupling. While the mode of action of Pep-1 has been extensively explored, the detailed molecular mechanisms responsible for cargo binding and translocation have still not been clearly identified. In this work, we present a Brownian dynamics (BD) simulation study using a coarse-grained model of Pep-1.

The equilibrium and dynamic properties of the peptide in (1) solution and (2) model membrane systems (e.g. DPPC and POPC bilayers) were monitored using simple mean-field potentials. The simplicity of the model employed allowed for 1 μs simulations to be performed. With the appropriate choice of parameters, the Pep-1 equilibrium and dynamical properties were recovered from the simulations and then compared with experimentally determined values.

Synthesis and biological evaluation of analogs of the A2 subunit (sequence 558–565) of the blood coagulation factor VIII

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Coagulation of blood is a complex process during which blood forms clots. This formation is an important part of hemostasis wherein a damaged blood vessel wall is covered by a platelet and fibrin-containing clot to stop bleeding and repair the vessel. Disorders of coagulation procedure can lead to an increased risk of bleeding (hemorrhage) or clotting (thrombosis). The coagulation cascade of hemostasis has two pathways, the intrinsic pathway and the tissue factor pathway, known as the extrinsic pathway, which leads to thrombin formation.

Factor VIII (FVIII) is an essential glycoprotein (GP) for the coagulation cascade of blood clotting and plays a crucial role in the intrinsic pathway, because it acts as the co-factor of the serine protease factor IX activation. These two factors form the complex, which was formerly known as "tenase of the intrinsic pathway". Deficiency of either is associated with the bleeding diathesis. The sequence 558–565 of A2 subunit is the region in which the FVIII interacts with FIX[1,2] and has the following structure:

$$\mathbf{Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-Asn^{564}-Gln^{565}}$$

The present research project covers the synthesis on solid phase support, the identification and the biological evaluation of linear and cyclic peptides[3] and peptidomimetics, b ased of the above sequence of the A2 subunit, aiming at the inhibition of interaction of FVIIIa with FIXa, in order to suspend the platelets adhesion and furthermore the thrombin production.

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Oostatic peptides containing D-amino acids as inhibitors of insect reproduction

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Deteriorating effect of the C-terminus truncated tetra- (4P) and pentapeptide (5P) analogs of *TMOF* decapeptide H-Tyr-Asp-Pro-Ala-Pro_6-OH on ovarian development (i.e. oostatic effect) of several insect species, stimulated the synthesis of new analogues containing D-amino acids and analysis of their oostatic activity and metabolic degradation after application into the body of the flesh fly *Neobellieria bullata.* The introduction of D-amino acids to 5P chain resulted in a stronger oostatic effect, in comparison with the parent 5P. Apparently, this effect is evoked without involvement of specific receptors. Therefore, an autonomous function of ovaries in a process of active intake of oostatic peptide, via passing it through inter-spaces of the follicular cells, could be suggested. Due to the cleavage elimination of the peptide bonds that D-amino acids participate on, the D-isomers were degraded much slowly than the native 5P. The prolonged survival and function of the D-isomeric molecules in the flesh-fly body could result in more ovaries influenced during a period of their development. In such way, the oostatic effect may be enhanced.
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A peptidomimetic compound to protect cell from copper mediating amyloid-β toxicity

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According to the amyloid-β(Aβ) cascade hypothesis of Alzheimer's disease (AD), Aβ peptide harbors neurotoxic properties and the decelerated degradation of Aβ may be the prime reason for Alzheimer's disease (AD). Aβ neurotoxicity involves forming peptide assemblies and evoking the generation of free radicals by binding and reducing reactive metals such as copper. The degradation of Aβ decline in the early stages of AD progression and with aging. It has been reported that Aβ aggregation blockers and copper chelators could have therapeutic benefits for AD. We show a multifunctional hybrid compound utilizing KLVFF as the recognition moiety, with the apocyclen ligating to KLVFF as the chelator. The apocyclen in this small molecular can capture Cu bound to Aβ, become activated, and prevent both Aβ oligomerization and toxicity as well as cleave the peptide. The strategy yielding this peptidomimetic compound has the potential to provide therapeutics for AD and other misfolding protein diseases.

Anticoagulant activity of synthetic linear analogue peptides of 1811–1818 loop of the A3 subunit of the light chain A3–C1–C2 of FVIII blood coagulation

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Coagulation is a major defense mechanism against bleeding but certain events as damage to the vessel wall or changes in blood flow can produce changes in the processes of coagulation that result in the pathological event of thrombosis. Factor VIII is a critical member of the blood coagulation cascade. In the intrinsic pathway, FIXa assembles on the surface of activated platelets as part of the intrinsic tenase complex comprised of FIXa, FVIIIa, phospholipids and calcium ions. Factor VIII is synthesized as a 2,332 amino acid single-chain glycoprotein. The domain organization is typically characterized as A1–A2–B–A3–C1–C2. The mature protein has a molecular weight of $\sim$300 kDa composed of a light chain and a heavy chain.

Recent studies have identified the FVIII light chain region Glu1811–Lys1818 as being involved in FIXa binding and in the assembly of the FX-activating-FIXa/FVIIIa complex.

A series of eight peptide analogues of the 1811–1818 loop of the A3 subunit of the light chain A3–C1–C2 of FVIIIa were synthesized and examined for their anticoagulant activity.

The activated partial thromboplastin time (APTT) and the FVIII activity were measured in vitro for all these peptides, according to procedures reported. Among the peptides investigated here, the protected peptide Ac-ETKTYFWK-NH_2 presented a prolongation of APTT more than 6 s and a significant reduction of FVIII activity above 40%.
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Phospholipid linked peptide for ultrasound imaging of angiogenesis

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Tumor growth and proliferation require angiogenesis in which vascular endothelial growth factor receptor (VEGFR-2, KDR) plays a crucial role. Targeted ultrasound imaging employing microbubbles bearing a peptide or an antibody, has been demonstrated in animal models. The most common method for preparation of targeted microbubbles is treatment of a streptavidin-coated microbubble with biotinylated targeting vectors. In order to optimally employ KDR targeted microbubbles in humans we sought to prepare phospholipid-based microbubbles bearing a KDR targeting ligand. The process for incorporation of a targeting vector into the phospholipid based microbubbles requires a phospholipid conjugate of the KDR targeting group. Using methods developed in our laboratories for the dimerization of biologically active peptides, a heterodimeric peptide with high binding affinity to KDR was prepared. Methods for the preparation of this high purity KDR targeting heterodimer peptide, its conjugation to a PEG linked phospholipid and its isolation employing eluent systems without trifluoroacetic acid are reported. A microbubble formulation incorporating the KDR targeting peptide-

phospholipid conjugate was successfully used for ultrasound imaging in an in-vivo angiogenesis model.

Isolation of an iron-binding peptide from animal blood plasma protein hydrolysates

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As an essential mineral, iron can be supplied in salts, elemental iron, metal chelates, and iron- binding proteins or peptides. Despite being the most popular, iron salts have low bioavailability in the body because of their reactivity with other food components. On the contrary, iron-chelated peptides increase the stability, absorption, and bioavailability of iron. Thus, peptide-chelated iron may be a suitable candidate as a supplement for improved iron absorption in the body. Therefore, the objectives of this study are aimed toward the development of porcine blood protein as a food ingredient and iron supplement, and the isolation of the iron-binding peptide from its hydrolysates. Porcine plasma protein was hydrolyzed using a commercial protease. The degree of hydrolysis and iron binding capacity were determined using the trinitrobenzenesulfonic acid and orthophenanthroline method, respectively. The hydrolysates of blood plasma protein were then filtered using YM-3 membrane, and an iron-binding peptide was isolated using gel permeation, ion exchange, and normal phase high-performance liquid chromatography. A novel iron-binding peptide from PBPP hydrolysates was purified and identified using liquid chromatography/electrospray ionization tandem mass spectrometry. The purified iron-binding peptide was identified to be a nonapeptide, Asp-Leu-Gly-Glu-Gln-Tyr-Phe-Lys-Gly (1,055 Da) based on mass spectrum and a sequence of porcine plasma protein. This is the first report regarding the isolation of an iron-binding nonapeptide from porcine blood plasma protein. These results suggest that the iron-binding peptide has a promising potential for the manufacture of functional food products as a supplement to iron.

Phosphorylation

Phosphoproteins on outer surface: a new target for controlling male fertility

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Sperm cells are the haploid male gametes and they are unique microscopic motile cells that perform a unique function in biology: fertilization of ova. Sperm forward progression and maturation are established as an interactive process between the cell and its surrounding environment. The flagellar beat kinematics, sperm morphology and surface properties are responsible for the rate of forward progression. Since 1976 many studies have been reported on the occurrence of ecto protein kinases and phosphoproteins in a variety of cell types including spermatozoa but their precise biochemical identities are largely unknown. The ecto-CIK is a membrane specific novel multipotential dimeric enzyme responsible for the regulation of a no of cellular functions by modulating phosphorylation of membrane bound proteins. This multipotential enzyme can be targeted as regulator of male fertility.

Immobilized GSK-3 kinase and MAP kinase for modification of phosphopeptides

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Attachment of phosphokinases GSK-3 kinase and MAP-kinase to magnetic microparticles carriers brings not only the advantage of an easy and repeated using but also non-contaminating and very specific and sensitive reaction on their substrates, i.e. phosphopeptides or phosphoproteins.

The binding method of choice was to immobilize enzymes covalently by different methods, using particles with carboxylic, PGL, amino-, hydroxyl- and epoxy-functional groups, which showed enzyme activity close to zero. Reasons for failure were composed of non-oriented immobilization strategy and/or inactivation of the enzyme by reactive chemicals. As alternative proved was the attachment the enzymes tagged with His·Tag by strong affinity interaction to IMAC carrier SiMAG-IDA/Nickel.

The immobilized GSK-3 was proved by applying the enzyme reactor for transferring the phosphate group to Ser7 of CREB phosphopeptide, in the mixture containing ATP. The substrate for immobilized MAP (ERK2) kinase used was the tyrosine hydroxylase sequence 24–33.

Three methods based on different principles were utilized for confirmation of immobilized phosphokinases functioning. By the first one, the MALDI-TOF mass spectrometry, we confirmed the 79 Da mass shift of the substrate in one phosphate group. For the selective detection of phosphoproteins separated by polyacrylamide gel electrophoresis the Phos-Tag^{TM} Phosphoprotein Gel Stain kit has been used. Beside this method based on selective enrichment of phosphopeptides using titanium dioxide (Phos-trap^{TM}) was utilized for isolation of peptides modified by GSK-3 and MAP kinases.

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The phosphoproteome of Fusarium graminearum at the onset of nitrogen starvation reveals phosphopeptides from proteins with a potential role in mycotoxin regulation

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The steps comprising the synthesis of deoxynivalenol (DON) and its derivatives by *Fusarium graminearum* are well understood, but its regulation at the molecular level is not. We are investigating a potential role for protein phosphorylation in initiating DON synthesis during nitrogen starvation in vitro. Multidimensional separation and analysis ('GeLCMS') was used to probe the phosphoproteome of *F.*

graminearum after the onset of DON synthesis. Proteins were first separated by SDS-PAGE; following a trypsin digest, peptides were extracted from gel slices and phosphopeptides enriched from these by a combination of immobilized metal affinity nano-scale chromatography and titanium dioxide affinity chromatography. Enriched samples were analyzed by LC–MS using a tandem mass spectrometer performing neutral loss scans on the most intense ions in the eluant to facilitate the detection and identification of phosphopeptides. The Mascot search engine was used to query the *F. graminearum* database for the identification itself. Over 200 phosphopeptides 30 samples from *F. graminearum* grown on nitrogen-poor media at $t = 0, 6$ h and 12 h (10 gel slices per time-point). Phosphorylation sites were assigned to all of them and confirmed manually by observing the presence of a neutral loss of 98 in the mass spectra. The peptides were from proteins involved in the regulation of protein synthesis, general metabolic enzymes, biosynthetic enzymes and proteins of unknown function. Many contained consensus kinase sequences. The biological role of some of these proteins in regulating DON synthesis will be assessed *in vivo* by producing *F. graminearum* mutants and measuring both their virulence and ability to produce DON.

Polyamines

Polyamine importers, including a novel putrescine importer, required for swarming induced by extracellular polyamines in *Escherichia coli* K-12

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Recently, many studies have reported that polyamines play roles in cell-to-cell signalling processes in bacteria. This presentation shows polyamine importers, including a novel putrescine importer, required for inducing swarming motility. The swarming phenotype of the *Escherichia coli* Delta-*speAB* Delta-*speC* strain, which cannot produce putrescine, is dependent on spermidine and PotABCD, a spermidine importer which was discovered in the 1990s. Moreover, the swarming motility of the *Escherichia coli* Delta-*speAB* Delta-*speC* Delta-*potABCD* strain, which cannot produce putrescine and cannot import spermidine from medium, was induced by putrescine supplemented to the medium. The responsible putrescine importers were searched, but introduction of the deletion of genes encoding known polyamine importers, Delta-*potE*, Delta-*potFGHI*, and Delta-*puuP*, and introduction of the deletion of genes encoding putative polyamine importer, Delta-*ydcSTUV*, into the Delta-*speAB* Delta-*speC* Delta-*potABCD* strain did not affect swarming induction by putrescine. The deletion of *yeeF*, a homologue of *puuP* and annotated to be a putative putrescine importer, in the Delta-*speAB* Delta-*speC* Delta-*potABCD* Delta-*ydcSTUV* strain abolished the swarming motility in putrescine-supplemented medium. The complementation of *yeeF* by a plasmid vector restored the swarming motility. The transport assay and the analysis of polyamine concentration in cells of the strains with *yeeF*$^+$ or Delta-*yeeF* in the Delta-*speAB* Delta-*speC* Delta-*potABCD* Delta-*ydcSTUV* genetic background revealed that YeeF was a novel putrescine importer.

Oxidation and degradation of γ-glutamylputrescine synthetase in Escherichia coli K-12

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γ-Glutamylputrescine synthetase (PuuA) catalyzes ATP-dependent γ-glutamylation of putrescine and PuuA is a part of putrescine metabolic pathway (Puu pathway) in *Escherichia coli*. The previous study shows that PuuA and glutamine synthetase (GS) of *E. coli* have several similar properties. For example, the amino acid sequences of PuuA and GS show high similarity. Moreover, the molecular weights of the monomers of the two enzymes are similar, and they exist as dodecamers. GS has a very interesting character. When GS is taken metal-catalyzed oxidation, the oxidative modification of GS plays a role of signal for degradation and GS is degraded. The two amino acid residues of *E. coli* GS which are important for metal-catalyzed oxidation of this enzyme are conserved in PuuA. Therefore, PuuA was supposed to have the same oxidation and degradation system as GS. In this study, metal-catalyzed oxidation and degradation of PuuA was examined using $FeCl_3$ and DTT as a metal-catalyzed oxidation system. When a purified PuuA was treated with $FeCl_3$ and DTT, the enzyme activity was lost gradually. The cell-free extract was also oxidized with $FeCl_3$ and DTT, and the stability of PuuA was monitored by Western blotting. As a result, when the cell-free extract was oxidized, the band of PuuA disappeared. This indicates that oxidized-PuuA was degraded by some proteases in the cell-free extract.

Supported liquid membrane extraction of polyamines from urine and other biological samples: novel way of sample preparation prior HPLC

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Polyamines are small, aliphatic amines involved in neoplastic process. Their elevated concentrations were found in tissues and urine of many cancer patients. Therefore they are considered as unspecific tumor markers and their determination in biological fluids possesses diagnostic relevance. Unfortunately polyamines pose many analytical problems. They are unstable, very polar compounds without chromophore groups what makes their analysis not so straightforward. The commonly applied liquid-liquid extraction is tedious, non-ecological, non-economic technique and above all is very labor-consuming. Therefore, new simpler methods are sought, ideally—with the potential to full automatization. Membrane-based extraction techniques are attractive alternative to traditional ones. In supported liquid membrane (SLM) the organic extractant liquid is immobilized in pores of polymeric support. The SLM extraction combines extraction of an analyte to organic phase and its reextraction to the aqueous phase in one process. The technique is cheap, leaves extremely little organic liquid waste, consumes little labor and time. Moreover it enables simultaneous clean up and enrichment of analyte.

In our work we investigated the SLM extraction of polyamines from urine and other biological samples followed by HPLC analysis. We optimized the extraction parameters and compared two different membrane geometries (planar and hollow fiber) along with two different systems of extraction (dynamic and static). It appeared that planar membrane in flowing system enables more efficient extraction

of polyamines than hollow fiber, but gives less enrichment. Nevertheless, both studied SLM extraction systems offer an interesting alternative to traditional liquid–liquid extraction for the extraction of polyamines from biological samples.

Proteins and proteomics

The reproductive role of multiple functional sites on P12, a Kazal-type trypsin inhibitor from male accessory sexual glands of mice

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We have demonstrated P12 (Spink 3: serine protease inhibitor, Kazal type 3), a 6-kDa caltrin-like protease inhibitor, in the mouse seminal vesicle secretion, and identified the reactive site (R^{19}) for its inhibitory effect on trypsin, the active site (Y^{21} or/and D^{22}) for the binding to the plasma membrane overlaying the anterior zone of intact spermatozal acrosome, and three epitopes on the N-terminal region, $^{43}RKR^{45}$, and the C-terminal region. The three epitopes on one sided are three-dimensional distant from R^{19}, Y^{21}, and D^{22} on the P12 molecule. We have determined a K_i value of 0.15 nM in the P12-trypsin interaction and a single type P12-binding site on mouse sperm (1.49×10^6 sites/cell) with a K_d value of 70 nM. Further, our recent study manifests two significant findings in mammalian reproduction: (1) P12 itself induces neither the capacitation nor the acrosome reaction of sperm and gives no impact on the protein tyrosine phosphorylation associated with the sperm capacitation, but it can greatly suppress the acrosome reaction induced by BSA, ionophore A23187, or both together. Such an inhibitory effect of P12 accompanies with a decrease in the intracellular [Ca^{2+}] in the space between the plasma membrane and the outer membrane of acrosome; (2) The P12-binding zone remains on the sperm in the uterine lumen but disappears on the sperm in the oviduct after the coitus. The presence of P12 even lowered to 12 μM in the incubation for in vitro fertility assay results in a great reduction of fertility. These data together manifested an indispensable mechanism of removing P12 from sperm before the egg-sperm encounter in the oviduct for fertilization under naturally coitus. The serine-type protease activity in the uterine fluid but not the BSA-induced capacitation is involved in the mechanism.

Purification and characterization of immunogenic proteins from the cattle tick, *Boophilus annulatus*

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Identification of antigens that induce an immune response against tick infestations is required for the development of vaccines against these economically important ectoparasites. In order to identify protective antigens, two specific larval glycoproteins (GLPs) were isolated from the larval stage of the cattle tick, *Boophilus annulatus* (Acari: Ixodidae), by two-steps affinity chromatography. The larval immunogens were first purified using CNBr-Sepharose coupled to rabbit anti-larval immunoglobulins, and the glycoproteins were then purified with Con-A Sepharose. These glycoproteins have molecular weights of approximately 32 and 15 kDa with isoelectric points between 6.8 and 7.2. Antibodies against the two GLPs, labeled I and II, were detected in the anti-whole tick, - whole larval, and -gut antigens through immunoblot analysis. These results suggest that these GLPs are good immunogens and can be useful in the vaccination of cattle against tick infestation.

The effect of crowding agent on the rate of aggregation of denatured α-lactalbumin

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Abstract: The structurally destabilized protein can aggregate and precipitate out of solution. The sHsp protein, α-crystallin, acts as a molecular chaperone stabilizing target proteins under stress conditions through the formation of a soluble complex. In this study, the effects of α-crystallin during its interaction with structurally destabilized α-lactalbumin, in the presence of the macromolecular crowding agent, dextran, have been examined. The interaction between the proteins was investigated by visible absorption spectroscopy, intrinsic fluorescence spectroscopy, ANS fluorescence binding and ^{1}H NMR (nuclear magnetic resonance) spectroscopy. In the presence of dextran, the rate and extent of aggregation of target protein was enhanced. α-Crystallin prevented aggregation of target protein but not as well as in the absence of dextran. It is proposed that a dextran-induced changes to protein conformation and α-crystallin was less effective in preventing the aggregation and precipitation of target proteins.

Analysis of isoforms of calcium independent phospholipase A2 in human platelets

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The phospholipases A2 (PLA2) are large family of enzymes classified in many groups and subgroups. These enzymes catalyse the hydrolysis of the sn- acyl-ester bonds of phospholipids of cell membrane, releasing free fatty acids and lysophospholipids. The calcium independent phospholipases A2 of group VI (iPLA2 G VI) are involved in remodeling of phospholipids of cell membrane, and pretty expressed in brain tissue and related to neurodegenerative diseases, such as schizophrenia and Alzheimer's disease. Previous reports have showed that the activity of PLA2 in human platelets is increased in schizophrenic patients and decreased in patients with Alzheimer's disease when compared with controls. The cloning of human cDNA of iPLA2 gene showed that multiple splice variants are generated from a single gene thus a new mechanism was proposed for their activity. The purpose of this work is the separation and identification of isoforms of iPLA2 GVI in human platelets using two-dimensional electrophoresis, immunodetection and mass spectrometry. Our results have showed that conditions of extraction of proteins may impair the iPLA2 precipitation. The best conditions of sample preparation have been chloroform/methanol

precipitation either folding or unfolding conditions. Acetone precipitation has been effective only under folding conditions. So far, two-dimensional separation has not been carried out because iPLA2 isoforms have not been focused therefore it has not been transferred to gel of the second dimension. Currently, we have been verified that isoforms focusing have been improved by the addition of more percentage of DTT and IPG buffer in re-hydratation solution.

Hydroxamic acids, novel inhibitors of serine racemase, interact with pyridoxal-5′-phosphate

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Mammalian serine racemase (SR) is a pyridoxal-5′-phosphate (PLP) dependent enzyme responsible for production of the neurotransmitter D-serine, which activates NMDA receptors in the CNS. As such, SR could be involved in glutamatergic neurodegenerative cascades. Recent results by Inoue et al. show that SR knock-out mice indeed have reduced NMDA and β-amyloid neurotoxic effects. The role of NMDA receptors in schizophrenia has also been generally accepted. Moreover several studies reveal correlations of D-serine metabolism and schizophrenia. Inhibitors of SR would therefore be a worthwhile tool for further studies in the fields. So far there are two known potent competitive inhibitors, L-*erythro*-3-hydroxyaspartate and malonate, with K_I values of 43 and 71 μM, respectively.

Here we present results from further screening of small molecule analogues for their SR inhibition. We introduce several new hydroxamic and dihydroxamic acid inhibitors; the most potent inhibitor ever described is succinodihydroxamic acid with the K_I of $(3.6 \pm 0.6)\mu M$ To our surprise, some of the active compounds, including succinodihydroxamic acid, act as both competitive SR inhibitors with K_I in low μM range and, at higher concentrations, as irreversible nonspecific inhibitors of PLP-dependent enzymes. We employed NMR, MS, and UV/Vis spectroscopic techniques to reveal that the irreversible nonspecific effect could be due to interaction of the hydroxamic acid moiety with PLP.

The loss of interaction to serine *O*-acetyltransferase (SAT) in some members of the OASS (*O*-acetylserine sulfhydrylase) family may be one of the important mutations leading to their differentiation into CAS (β-cyanoalanine synthase) during evolution

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CAS and OASS are belonged to the β-substituted Ala synthase (Bsas) family and demonstrated to have overlapping catalytic activities in cyanide detoxification and cysteine synthesis in vitro. However, the CAS activity must evolve later because the CAS must rely on the cysteine supply from OASS. CAS is a unique member in rice mitochondria; while OASS with at least three members localize to cytosol, plasmids and mitochondria in Arabidopsis. It is doubtful whether OASS can work properly in an alkaline environment like mitochondrial matrix, but CAS can maximize its activity at this pH and mitochondrial matrix also act as an alkaline trap for cyanide. It is possible that CAS evolved from a mitochondrial OASS to remove cyanide derived from cyanogenic compounds including 1-aminocyclopropane-1-carboxylic acid during ethylene biosynthesis, so as to protect the electron transport during respiration.

The roles of amino acid residues on rice CAS were investigated by mutagenesis in a yeast expression system. The specific activity of the wild type rice CAS was determined to be 111.3 μmol H_2S/min/mg protein. A mutation on putative co-factor pyridoxal 5′-phosphate (PLP) binding Lys^{99} caused a loss of CAS activity; mutations on Lys^{120} and Lys^{168} to Glu decreased an affinity of cyanide binding significantly; a mutation on Leu^{275} to Lys to re-build the putative SAT interacting interface in rice CAS caused a significant drop in specific CAS activity. The last mutagenesis result suggests that the loss of SAT interacting domain in OASS would be an important event leading to CAS differentiation during evolution.

Functional roles of hinge region, loops 3 and 4 in the modular halves of *Escherichia coli* cyclic AMP receptor protein

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Escherichia coli cAMP receptor protein (CRP) is a homodimer in which each subunit is composed of two functional domains. The C-terminal domain is responsible for DNA recognition, whereas the N-terminal domain is involved in cyclic nucleotide binding which leads to the activation of CRP. The hinge region that links the two function domains plays a pivotal role in the allosteric signal transmission pathway, whereas loop 3 (between β-strands 4 and 5) and loop 4 (between β-strands 6 and 7) are involved in global structural perturbations such as subunit realignment and domain rearrangement. To test the functional roles of the hinge region, loop 3 and loop 4 in the intersubunit interaction in CRP and effects of these two loops on CRP's biological functions, WT CRP, insertion and insertion-deletion mutants were studied using two proteolytic fragments of these proteins. Subtilisin digestion produces a fragment (S-CRP) of CRP with residues 1–134, while chymotrypsin digestion produces a fragment (CH-CRP) of CRP consisting of residues 1–137 with a hinge region tagged at the end of C-helix. All results indicate that the allosteric signal transmission process that induced by cAMP is most likely a result of overall realignment of the two subunits within a CRP dimer.

Identification of cPKCgamma interacting proteins involved in cerebral ischemic/hypoxic preconditioning

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Cerebral ischemic/hypoxic preconditioning (I/HPC) is an endogenous protective phenomenon which protects brain against subsequent severe ischemia/hypoxic injuries. Using our established autohypoxia-induced I/HPC mouse model. We found that conventional protein kinase C (cPKC) gamma (γ) activation is involved in the

neuroprotective effect of I/HPC against middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemic injury. However, the alteration of cPKCγ interacting proteins in brain is unknown. In this study,we explored the changes in cPKCγ interacting protein expressions after I/HPC and ischemia by using comparative proteomic approaches. Adult male BALB/C mice were assigned into four groups as follows: sham, ischemia, I/HPC+ ischemia with or without cPKC inhibition (Go6983) groups. Tissue extracts from cortex were subjected to two-dimentional electrophoresis, and the gel spot patterns were analysed by using the 2D Platinum software. Totally, nineteen protein expressions changed significantly in I/HPC+ ischemia group when compared with ischemia group. Among them, the changes of eight proteins could be abolished by cPKCγ inhibition, which including dynamin-1, HSP60, HSP70, lactoylglutathione lyase, peroxiredoxin 2, γ-enolase, ubiquitin carboxyl-terminal hydrolase isozyme L1 and NADH dehydrogenase [ubiquinone] flavoprotein 2. Immunoprecipitation study showed that cPKCγ can directly interact with these proteins except lactoylglutathione lyase and peroxiredoxin 2. These proteins are involved in biological processes including cytoskeleton, chaperon, stress/antixoidants, metabolism, ubituitin proteasome system and oxidation phosphorylation. Taken together, we firstly identified the cPKCγ signaling related proteins involved in the development of cerebral I/HPC. Further studies will be focused on the effects of these differentially expressed proteins on focal cerebral ischemic injuries.

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Molecular cloning and sequencing of Penicillin acylase enzyme from *Shigella boydii*

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Biology is a rich science regarding advances in experimental techniques that produce various proteins and other materials. The resulting product, 6-aminopenicillanic acid (6-APA), is utilized in the production of a variety of semi synthetic Penicillin. Formation of active PGA includes a series of post translational steps via translocation and periplasmic processing. The production of PGA by recombinant protein technology can produce PGA enzyme more and cheap. The aims were: (1) screening of non *E. coli* members of Enterobacteriaceae from environmental and clinical specimens for PGA by PCR. (2) PGA cloning from non *E. coli* bacteria and sequencing of the gene. (3) Expressing of cloning PGA in the *E. coli*.

In this study, 290 non *E. coli*, Enterobacteriaceae were isolated from environmental and clinical specimen. A PGA positive strain (*Shigella boydii*) was isolated. DNA was separated from this isolate and was entered in PCR reactions using primers designed on conserved region of PGA genes. The PCR product was cloned in pGEM-Teasy vector. Sequencing of cloned portion indicated that the gene encoding Penicillin G acylase from *Shigella boydii* included an open reading frame of 2538nucleotide encoding 846 amino acids. Survey of sequencing result revealed that this gene contain 98% homology to previously reported PGA from a *Shigella boydii* strains. Results of this study indicated that the polymerase chain reaction method is a very sensitive and specific method that can be utilized for screening of PGA genes from different samples.

From MALDI high energy CID (collision-induced dissociation) for structure elucidation of biomolecules to MALDI imaging mass spectrometry of biological surfaces

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Peptides and lipids have been analyzed extensively so far by various desorption/ionization techniques as, e.g. FAB, ESI and MALDI. Tandem and multistage mass spectrometric approaches for detailed structural investigation utilizing CID of these analytes were performed mainly on triple quadrupole instruments, IT, hybrid devices (Q/RTOF), IT/RTOF), sector instruments, or, more recently, on TOF instruments. Whereas the former three types of instruments allow only low-energy CID (<200 eV collision energy), the latter two are operated under high-energy CID conditions (1 up to 20 keV). Characteristic features for low-energy CID spectra of biomolecules are fragmentations related to carbon-heteroatom cleavages. For high-energy CID spectra additional carbon-carbon cleavages, requiring typically higher collision energies, are observed. Especially TOF/RTOF instruments fitted with a grounded or floating collision cell and differential pumping offer the possibility of vacuum MALDI in combination with true high-energy CID in the 20 keV regime thus allowing detailed structural analysis (e.g. leucine/isoleucine, double bond location in fatty acids) To demonstrate the influence of the selected, quite different collision energies on the appearance of resulting CID spectra two different MALDI-TOF/RTOF- and one MALDI IT/RTOF-instrument were used: the ABI 4700 (1 keV), the Shimadzu TOF^2 (20 keV) and Shimadzu QIT (4–100 eV). Of particular focus will be the information content of the individual CID data for structure determination of unknowns in "omics"-related research. Finally, MALDI TOF/RTOF- and Q/RTOF-MS will be used to perform imaging MS of small proteins directly from a biological surface as natural latex to detect proteinuous allergens and determine their lateral distribution.

Proteomic analysis of livers from a transgenic mouse line with activated polyamine catabolism

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We have generated a transgenic mouse line that overexpresses the rate-controlling enzyme of the polyamine catabolism, spermidine/spermine N^1 acetyltransferase, under the control of a heavy metal inducible promoter (MT-SSAT). This line is characterized by a notable increase

in SSAT activity in liver, pancreas and kidneys and a moderate increase in the rest of the tissues. SSAT induction results in a enhanced polyamine catabolism manifested as a depletion of spermidine and spermine and an overaccumulation of putrescine in all tissues. In order to study how the activation of polyamine catabolism affects other metabolic pathways, protein expression pattern of the livers of transgenic animals was analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS).

A total of 23 proteins were shown to be differentially expressed in the transgenic and wild-type animals. Many of the identified proteins showed expression patterns expected associated with polyamine catabolism activation. However, the expression pattern of other proteins, such as repression of GST pi and selenium binding protein 2 and 60 kDa heat shock protein, could be explained by the overexpression of peroxisome proliferator-activated receptor γ co-activator 1 α in response to depleted ATP pools. The activation of the latter proteins is thought to lead to the improved insulin sensitivity seen in the MT-SSAT animals.

Characterization of the interaction between human serum albumin and paclitaxel: Spectroscopic approaches

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The interaction of paclitaxel with human serum albumin (HSA) has been investigated by using fluorescence, UV absorption and circular dichroism spectroscopic approaches for the first time. Fluorescence data revealed the presence of a specific binding site on HSA for paclitaxel and the binding affinity was $2.41 \pm 0.17 \times 10^{-5}$ L mol^{-1} in the physiological condition. The HSA-paclitaxel interaction caused an obvious red shift of the UV absorption band I of paclitaxel and paclitaxel bound to the protein as an anionic species with deprotonation of one hydroxyl group on B ring in the physiological condition (pH 7.4). It was also noticed that the level of protonation of the hydroxyl groups played an important role during the drug-protein. The results of synchronous fluorescence spectra and three-dimensional fluorescence spectra showed that binding of paclitaxel to HSA can induced conformational changes in HSA. The interaction between paclitaxel and HSA induced an obvious reduction of the protein alpha helix and beta-sheet structures. The second derivative of HSA fluorescence spectra in the presence of paclitaxel were characterized in order to describe changes in the tryptophan environments of proteins. The relative peak composition of the HSA derivative spectra excited at 295 nm is indicative of the rather hydrophobic environment of its tryptophan residue ($H = 0.95$), as expected from HSA structures. On the other hand, excitation of samples at 280 nm revealed the influence of the high tyrosine content of HSA in the region of emission below 320 nm.

TAP-proteomics to study GPCR signaling: The interactome of the adenosine-A2A receptor

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The A_{2A}-receptor is a prototypical G_s-coupled receptor, which has several unique structural and functional characteristics. In contrast to the classical model of collision coupling described for the β-adrenergic receptors, the A_{2A}-receptor couples to adenylyl cyclase by restricted collision coupling and forms a tight complex with G_s. The A_{2A}-receptor has a long C-terminus (of >120 residues), which is for the most part dispensable for coupling to G_s. It was originally viewed as the docking site for kinases and the β-arrestins, which initiate receptor desensitization and endocytosis. The A_{2A}-receptor is, however, fairly resistant to agonist-induced internalization. Recently, the C-terminus has been appreciated as a binding-site for several additional "accessory" proteins, of which only few are established.

Our aim is to document which of these interactions do occur in living organisms by characterizing the interactome of the A_{2A}-receptor using a two-step proteomics approach: we expressed tagged receptors in cells using various TAP-tag variants; this allowed for characterization of the expression and functionality of the modified (tagged) receptors, optimization of the purification conditions and an initial survey of interaction partners using a 2D-nano-LC–MS/MS approach. Subsequently we are generating knock-in mice to afford the isolation of receptor complexes from native tissues.

This approach allows for testing which protein-complexes (signalosome) are preferentially formed under specific conditions and their analysis may not only aid the development of treatments for A_{2A}-receptor related diseases, but provide the proof-of-principle for the entire family of GPCRs.

Identification and characterization of serotonin receptors by gel-based proteomics

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Even though conventional two-dimensional gel electrophoresis is widely used for proteomics, highly insoluble transmembrane proteins are still limited for application because of their high insolubility, lipids and other artifacts. And its chaotropic environment makes hydrophobic membrane proteins difficult to analyze for their complex constituent and binding partners. Detergents play important roles for isolation, solubilization and manipulation of membrane proteins and their subsequent biochemical and biophysical characterization. We can analyze insoluble hydrophobic membrane proteins by combining appropriate detergents with various separation methods coupled with mass spectrometry. The serotonin receptor is an important G-protein coupled neurotransmitter receptor involved in various roles such as behaviors, cognitive function and development. These hydrophobic transmembrane neurotransmitter receptors are also difficult to isolate, solubilize and identify in a native form via conventional gel electrophoresis. We used discontinuous gel electrophoresis using Coomassie staining dye and nonionic detergent Triton X-100, dodecyl maltoside, digitonin with subsequent one- or two-dimensional SDS-PAGE step using different gradient polyacrylamide gel electrophoresis system followed by mass spectrometry. It was the aim of study to separate and identify serotonin receptors and its binding partners from mice hippocampi. For this experiment samples were homogenized, soluble proteins were discarded by ultracentrifugation and the water-insoluble membrane-enriched pellet was resuspended in a mixture containing 6-aminocaproic acid, Bis-Tris and nonionic detergents. Blue Native PAGE (BN-PAGE) was performed under cooled condition without SDS. BN gel strip containing separated proteins and protein complexes was excised and re-run on SDS-PAGE. Proteins were identified and analyzed by nano-HPLC-ESI-(CID/ETD)-MS/MS.

Gel-based mass spectrometric analysis of a strongly hydrophobic GABAA receptor subunit containing four transmembrane domains

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Analysis of highly hydrophobic proteins is still an analytical challenge. Using a recombinant $GABA_A$ receptor subunit as a model protein, a gel-based proteomic approach for high MS/MS-peptide sequence coverage-identification was developed.

Protein samples were separated by multi-dimensional gel electrophoresis: blue native gels were used to obtain bands representing receptor complexes followed by one-dimensional SDS-PAGE of individual excised bands that were finally run on two-dimensional SDS-PAGE to obtain optimal separation of protein spots. The protein spot representing the $GABA_A$ receptor subunit alpha-1 from the last electrophoretic step was used for in-gel digestion with trypsin, chymotrypsin and subtilisin, followed by subsequent mass spectrometric identification by nano-ESI-LC–MS/MS QstarTM XL and linear ion trap LTQ XL identification.

The protein was unambiguously identified with 100% sequence coverage, thus covering all four hydrophobic transmembrane domains. Development of gel-based mass spectrometric analysis may represent a step forwards forward in the complex analysis of other membrane or hydrophobic proteins.

Proteomic analysis of larvae during metamorphosis in the spionid polychaete *Pseudopolydora vexillosa*

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Metamorphosis in marine invertebrates is a critical process that will subsequently impact their recruitment success and the population dynamics. Yet the molecular mechanisms behind metamorphosis remain largely unknown. Proteomic technologies have been widely used in model organisms to detect protein expression profile changes, but their applications on marine organisms are at an early stage. As part of our study to understand the mechanisms behind larval metamorphosis of a polychaete (*Pseudopolydora vexillosa)*, we analyzed the proteomes during metamorphosis using two-dimensional gel electrophoresis. We compared the global protein expression patterns of pre-competent and competent larvae (those ready to settle and metamorphose), and newly metamorphosed juveniles. The proteomes of pre-competent larvae were significantly different from those of competent larvae and newly metamorphosed juveniles. We detected significantly fewer protein spots (384 spots) in pre-competent larvae than in the other two stages, while the proteomes of competent larvae and newly metamorphosed juveniles both expressed about 660 protein spots. Some proteins that were abundantly expressed and up-regulated during competence were identified using MALDI-TOF/TOF; the identified proteins included a molecular chaperone (calreticulin), a signal transduction regulator (tyrosin activation protein) and a tissue-remodeling enzyme (metallopeptidase). This is the first study of the proteome patterns present during larval development and metamorphosis in polychaetes. The similar protein expression profiles shows that further investigations on post-translational modifications is required to answer the morphological changes during metamorphosis.

Quantitative proteomics of ubiquitin pathways

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Protein ubiquitination plays an essential regulatory role within all eukaryotes. Large-scale analyses of ubiquitinated proteins are usually performed by affinity purification strategies coupled with mass spectrometry (MS). We have developed a series of methods to enrich, identify, and quantify ubiquitinated species, as well as to systematically remove co-purified false positives. Moreover, we have developed bottom-up and middle-down MS strategies to determine Ub-modified lysine residues and to analyze the structure of poly-ubiquitin chains. Using these MS-based proteomics tools, we have found that unconventional polyubiquitin linkages are unexpectedly abundant in cells and function in a variety of cellular processes.

Corneal proteomics: a better understanding of molecular mechanisms underlying epithelial wound healing

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Damage and injury of the corneal epithelium are key features of various corneal disorders and require constant repair of the epithelium. An intact epithelium of the cornea represents a crucial barrier to a variety of pathogens causing corneal complications. An impairment of the integrity of epithelium is observed in the course of various corneal disorders including road accident victims, industrial workers, trauma and post operative complications etc. Rapid recovery of epithelium following injuries is accomplished by a number of process but the mechanism and modulation of such processes are not yet clearly understood.

We have demonstrated here that re-epithelialization is regulated and modulated at both transcriptional and translational levels. Healing of corneal epithelium is regulated in fact by a complex network of highly divergent factors among them a large number of proteins that have been identified in epithelium which play an essential role in cell proliferation,

migration and cell matrix adhesion. Enhancement of epithelial repair mechanism by regulatory/modulatory factors may provide future approaches for the treatment of disorders that are characterized by injuries or delayed re-epithelialization of corneal surface.

Glutamate dehydrogenase; structure, evolution, regulation, and role in insulin homeostasis

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Glutamate dehydrogenase (GDH) is found in all living organisms but only animal GDH is regulated by a large repertoire of metabolites. More than fifty years of research to better understand the mechanism and role of this allosteric network has been frustrated by its sheer complexity. However, our recent structural and biochemical studies have begun to tease out how and why this complex behavior evolved. Much of GDH regulation likely occurs by controlling a complex ballet of motion necessary for catalytic turnover and evolved concomitantly with a long antenna-like feature of the enzyme's structure. *Ciliate* s, the 'missing link' in GDH evolution, might have created the antenna to accommodate changing organelle functions and was refined in humans to, at least in part, link amino acid catabolism with insulin secretion. The link between GDH and insulin homeostasis was clearly demonstrated by the finding that the genetic defects in children that led to a loss of allosteric inhibition of GDH by GTP results in a hyperinsulinism/hyperammonemia disorder where the children are particular sensitive to leucine, an activator of GDH. We have continued these studies by identifying a number of possible therapeutic agents, including polyphenols from green tea, that can inhibit even the mutated form of the enzyme.

Synthesis and antiviral activity of some analogues of acyclovir with cinnamic acids

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Following the discovery of the first effective antiviral compound (idoxuridine) in 1959, nucleoside analogues, especially acyclovir (ACV) for the treatment of herpesvirus infections, have dominated antiviral therapy for several decades. However, ACV and similar acyclic nucleosides suffer from low aqueous solubility and low bioavailability following oral administration. Derivatives of acyclic nucleosides, typically esters, were developed to overcome this problem and valaciclovir, the valine ester of ACV, was among the first of a new series of compounds that were readily metabolized upon oral administration to produce the antiviral nucleoside in vivo, thus increasing the bioavailility by several fold.

Cinnamic acids and their derivatives (esters, amides and glycosides) attract attention in biology and medicine because of their antiviral, antioxidant, anti-inflammatoryt, antimutagenic properties.

We synthesized and explored antiviral activity (HSV-1, HSV-2) of some analogues of acyclovir with cinnamic acid- sinapic, *p*-coumaric, ferulic acids. The derivatives of acyclovir were using *N*-ethyl-*N*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 4-*N*,*N*-(dimethylamino)-pyridine (DMAP).

The chemical stability of some of them was studied at pH 1.0 and 7.4 and 37°C. An HPLC method was developed for quantification of the ester concentration.

Importance of *cis/trans* isomerization of prolyl peptides for inhibition of Angiotensin converting enzyme

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Several synthetic drugs and peptides are widely available for inhibition of angiotensin converting enzyme (ACE) and posses antihypertensive effect. The tripeptides Val-Pro-Pro and Ile-Pro-Pro isolated from casein are one of the most promising compounds for prevention and treatment of hypertension in medicine and as a food additives.

The aim of the study is to obtain information about the active conformation in the recognition process of prolyl peptides as inhibitors of ACE. Both, classical and solid phase methods of peptide synthesis have been used for preparing of Val-Pro, Ile-Pro, Val-Pro-Pro, Ile-Pro-Pro, Val-Pro-Pro-Pro and Ile-Pro-Pro-Pro. For the synthesis of the target peptides, with free C-terminal group we used 2-clortrityl chloride resin and Fmoc strategy.

The conformation of prolyl peptides has been study by the methods of NMRspectroscopy—1H, 13C, COSY, DEPT, and NOESY. 1H NMR spectra of prolyl di and tri peptides as a function of pH (2.5–8.5), temperature (278–303°K) and solvent (D2O,DMSO-d-6 and CD3OD) have been recorded at 600 MHz and the equilibrium of *cis/trans* isomers has been determined. Some preliminary theoretical results, indicated that *trans* conformer of prolyl peptides as inhibitor of angiotensin converting enzyme is preferred to *cis*.

Molecular chaperones in ER-mitochondrial calcium signaling

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Ca^{2+} signaling between the endoplasmic reticulum (ER) and mitochondrion plays a pivotal role in bioenergetics, metabolism, and survival. The ER-mitochondrion interface known as the mitochondrion-associated ER membrane (MAM) integrates multiple signaling pathways and plays a key role in the ER-mitochondrion Ca^{2+} signaling by serving as Ca^{2+} tunnels. Upon the activation of metabotropic receptors which induce an increase of IP3, Ca^{2+} is released from the ER via IP3 receptors (IP3Rs) at the MAM and then uptaken into mitochondria by the uniporter. An outstanding question is: IP3Rs are prone to protesomal degradation after opening, how does nature deal with this problem? By using CHO cells, we found that an ER resident protein called the sigma-1 receptor (Sig-1R), which is highly concentrated at the MAM, regulates the stability of IP3Rs by acting as a ligand-regulated receptor chaperone. Normally, the Sig-1R forms a dimeric complex with another ER chaperone BiP. However, by sensing the drop of ER lumenal Ca^{2+} or by the agonist-induced activation, the Sig-1R dissociates from Bip and begins to chaperone and stabilize IP3Rs. In Sig-1R-knockdown cells, IP3Rs are highly ubiquitinated and degrade rapidly. The Ca^{2+} signaling from the ER into mitochondria is attenuated in Sig-1R-knockdown cells. In addition, we found that, upon prolonged ER stress that causes apoptosis, Sig-1Rs

dissociate from BiP and translocate from the MAM into the entire ER reticular network to attenuate apoptosis. Results suggest that the ER is a signaling organelle regulating bioenergetics and survival via Sig-1R chaperones at the MAM.

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Enzyme-mediated site-specific labelling of proteins with a new multipurpose biotin tag

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Methods for labelling specific proteins using 'bio-orthogonal' chemistry have been a significant focus for research in recent years. Introduction of a chemical handle that can be selectively modified to incorporate a luminescent tag, crosslinking agent or to allow selective immobilization of a protein is of considerable use in the study of protein-protein interactions and protein trafficking within cells. A number of methods of introducing suitable chemical functionality in proteins are described in the literature including the use of un-natural amino acid mutagenesis; the feeding of azido-functionalized sugar precursors; and the use of biotin ligase to biotinylate lysine residues in specific 13–15 amino acid peptide sequences. The last of these methods has the advantage that it is practically simple to achieve using commercially available materials.

We have designed and prepared several new 'azido-biotin' compounds based on vitamin H. The ligation efficiency of the new biotin analogues to the 'Avitag' peptide sequence with BirA, the biotin ligase from *E. coli* has been determined along with the binding affinity of the new biotin analogues with avidin using ITC (Isothermal Titration Calorimetry). These compounds have moderate to strong binding affinity for avidin. The biotin analogues can therefore be used to reversibly immobilize the target proteins to avidin columns and released on addition of biotin. Alternatively, they can be irreversibly covalently modified via 'click' or Staudinger-Bertozzi chemistry to introduce fluorescent tags, crosslinking agents or immobilized covalently on to synthetic supports.

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Protein profiling of childhood acute myeloid leukemia

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Childhood acute myeloid leukemia (AML) is a frequent hematological malignancy with a high degree of heterogeneity in response to therapy. Given its diverse nature and its inherent variability in individual protein levels, it seems likely that the best approach to screen for AML is determine the protein profile using proteomics. We studied two cases of childhood AML to identify proteins, whose function could regulate the treatment outcome of the disease. By these, one was myelodysplastic syndrome (MDS) before AML diagnosis and the other AML. Six bone marrow plasma (BM) and six peripheral blood plasma (PB) samples were obtained before diagnosis, at diagnosis of AML and three months following initiation of treatment. As controls, two plasma samples from BM and two from PB from non-leukemic pediatric patients were studied. Differential proteomic analysis was performed by two-dimensional gel electrophoresis followed by protein identification by MALDI-TOF mass spectrometry. Comparison of plasma samples from BM and PB of the AML pediatric patients with the controls revealed that forty one proteins were found differentially expressed between the BM samples as compared to the control, whilst 26 were found differentially expressed between the PB samples as compared to the control. Functional analysis showed that the majority of the detected proteins involved metabolic enzymes, structural proteins, signal transduction mediators and immunoglobulins. Proteome analysis of plasma offered a useful approach for profiling pediatric patients with AML and provided significant insight into the presence and/or absence of several proteins that could serve as useful biomarkers for childhood AML.

Interactions between large and small subunits of different acetohydroxyacid synthase isozymes of *E. coli*

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Acetohydroxyacid synthases (AHASs) are thiamine diphosphate-dependent enzymes, catalyzing the first common step in the biosynthesis of valine, isoleucine and leucine. The three AHAS isozymes in enterobacteria, encoded by genes *ilvBN*, *ilvGM* and *ilvIH* (AHAS I, II and III, respectively), differ in kinetic properties and valine response. AHAS isozymes are composed of two kinds of subunits. The large catalytic subunits (LSU—ilvB, ilvG and ilvI), with molecular weights of 60–62 kD, have only 0.05–15% of the activity of the corresponding holoenzymes. The small regulatory subunits (SSU—ilvN, ilvM and ilvH), with molecular weights 9–17 kD, each associate with the corresponding large subunit and confer full catalytic activity and valine sensitivity to the holoenzyme (AHAS I and III).

Is any effective nonspecific interaction possible between LSUs and SSUs of different isozymes? To answer this question we have now examined the effects of each of the isolated SSUs on all of the isolated LSUs and found that the AHAS II SSU ilvM is able to activate the LSUs of all three of the isozymes, and the truncated AHAS III SSUs ilvH-Δ86 and ilvH-Δ89 are able to activate the LSUs of both AHAS I and AHAS III. However, none of the heterologously-activated hybrid enzymes have any feedback sensitivity.

HpaXm from *Xanthomonas smithii pv. smithii* is a novel harpin protein with signal peptide

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A novel harpin-like protein, hpaXm, was described from the cotton leaf blight bacteria, *Xanthomonas. smithii* subsp. *smithii* (*Xss*,a synonym of *Xanthomonas campestris* pv. *malvacearum Xcm*). Like harpins from other plant bacteria, the HpaXm encoded by a *hrp* gene was a glycine-

rich, cysteine lacking, heat-stable protein with the activity to induce hypersensitive reaction accompanied by enhanced expression of defense-related genes on tobacco leaves. The *hpaXm* was localized in between *hrp2* and *hrcC* by TAIL PCR technology. The *hrcC* from *Xss* shared 93% identity with that from *X.oryzae*.pv.*oryzae* (*Xoo*) and *hpa2* from *Xss* shared 89% identity with that from *Xoo* by DNA alignments. Two α-helix domains were present respectively at N- and C-terminal regions of hpaXm. Phylogenetic analysis with complete amino acids and 13 highly conserved amino acids H_2N-SEKQLDQLLTQLI-COOH in N-terminal α-helix regain indicated that hpaXm had an evolutionary relationship closer with hpaGXag and hpaXac than with hpa1Xoo and hpa1Xoc. A signal peptide was predicted at the 15 leading amino acids in the N-terminus and GST trap test suggested that the signal peptide may function to release hpaXm in the extracellular medium and the TypeII secretion system as sec-dependent pathway may associate to hpaXm secretion.

A study on metamorphosis of a marine invertebrate, the bryozoan Bugula neritina, by 2DE based proteomics approach

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Metamorphosis in benthic marine invertebrates involves permanent attachment of the larva to the substratum and the initial reorganization of the larval tissue followed by morphogenesis of adult tissue. Although many studies have documented ultrastructure changes, little is known about the molecular mechanisms involved in metamorphosis of marine invertebrates. The bryozoan *Bugula neritina* is a good model to study metamorphosis in marine invertebrates because initial reorganization of the larval tissue is both dramatic and quick, with total reorganization within 10 min, followed by further morphogenesis. We used 2DE-based comparative proteomic approach to study whether larvae are heavily engaged in protein synthesis during the early phase of metamorphosis. Surprisingly, the total proteome does not alter significantly. In more than 900 spots detected, only 20 and 23 spots are specific to swimming larval and early metamorphic stages, respectively. We then shifted our focus to protein phosphoylation, which is important for cell differentiation, cell migration and cell proliferation. Major shifts in phosphoproteome were observed, with more than 100 proteins specifically phosphoylated in early metamorphic larvae. The result suggested that post-translation modification may play a major role in larval tissue reorganization and morphogenesis during the early phase of metamorphosis.

2D gel based multiplexed proteomics approach to understand antifoulant's effects on the fouling barnacle cyprids (*Balanus amphitrite*)

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Macrofouling by the barnacle *Balanus amphitrite* and other benthic invertebrates causes severe economical impacts, leading to the growth of a biotechnology field focused on development of safe, exclusive, and effective antifouling agents. A variety of antifouling compounds have been shown to effectively inhibit barnacle larval settlement, but the molecular mechanisms behind their biological activity remains unclear. Recently, proteomics technology has enabled us to determine protein expression level alternation in response to treatment with antifouling compounds. As a further extension of this work, we employ a new 2-DE multiplexed proteomics technology (combination of Pro-Q Diamond with SYPRO Ruby dye) to elucidate the protein expression and phosphorylation changes that occur in when the organisms is treated with a settlement-inhibitive chemical cue. Both the protein and phosphoprotein profiles change between the antifoulant treatment and control groups. The 0 h and 24 h control barnacle larvae expressed about 303 and 357 phosphorylated proteins respectively, whereas only about 217 phosphoproteins were detected in larvae challenged with antifouling compounds. In addition, the ratio between phosphoproteins and total proteins changed in a similar trend. Overall, our results suggest that the level protein phosphorylation plays a crucial role during larval settlement and that the antifoulants tested inhibit larval settlement by alternating phosphorylation status that rather than affecting protein synthesis. We hope understanding the proteomic changes induced by antifouling agents will aid in the development of more effective and exclusive antifouling agents in the future.

Study of the importance of [ProB28] on the self association of insulin. Semisynthesis of des [ProB28] insulin

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In the past few years, many attempts have been made to prepare a synthetic insulin. The biological activity of insulin is known to be closely related to the C-terminal octapeptide fragment of its B-chain. This does not necessarily mean, however, that each of the amino acid residues of the octapeptide fragment is essential for its activity.

It would therefore seem desirable to study the importance of [Pro_{28}] residue B-chain on biological activity and self association of insulin des [Pro B_{28}]. The present work describes the preparation of the protected octapiptide derivative (23–30) B-chain human insulin des [Pro_{28}], by the solid phase peptide synthesis.

Gly	−phe	−phe	−tyr	−thr	−lys	−thr
23	24	25	26	27	29	30

Splitting same octapoptide $B_{23\text{-}30}$ from natural insulin by using trypsin giving insulin des octapeptide (DOI), by recupling of (DOI) to the synthetic octapiptide $B_{23\text{-}30}$ des Pro_{28}, by using trypsin in acidic medium giving [Insulin des (Pro B_{28})], which under investigation for self association and biological activity.

2D gel based multiplexed proteomics analysis of differentially regulated protein during larval development and settlement in the polycheate Hydroides elegans

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Larval settlement of biofouling organisms (benthic invertebrates that live on submerged man-made surfaces) is a focus of both ecological studies on colonization processes and applied studies on antifouling control. Larval settlement is a dynamic process that includes both behavioral and metamorphic changes that require tissue remodeling and differentiation in addition to a variety of biochemical and physiological alterations mediated by differential expression of genes and proteins. Although post translation modifications (PTM) such as protein phosphorylation, are crucial for variety of rapid cellular responses such as signal transduction and signal cascades, little is known of differential protein expression and PTM during larval settlement. In this study, 2-DE multiplexed proteomics technology (combination of Pro-Q Diamond with SYPRO Ruby dye) was used to monitor the changes in protein expression and phosphorylation during larval development and settlement in the common biofouling polychaete *Hydroides elegans*. The profiles of larvae before and after they are competent to settle were similar in terms of major proteins but the number of phosphorylated protein spots increased from 110 to 225. Both the protein and phosphoprotein profiles of adults were distinctly different from that of other life stages, with only 96 phosphorylated protein spots in adult stage. In addition, the ration between phosphoproteins and total proteins changed in a similar trend, indicating that the level protein phosphorylation might play a crucial role at the initiation of larval settlement. To our knowledge, this is the first study demonstrating that quantitative changes in the phosphorylation state of proteins involved in larval settlement.

Hippocampal protein levels related to spatial memory are different in the Barnes maze and in the multiple T-maze

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The multiple T-maze (MTM) and the Barnes maze (BM) are landmazes used for the evaluation of spatial memory. The observation that mice are performing differently in individual mazes made us test the hypothesis that differences in cognitive performance in the two landmazes would be accompanied by differences in hippocampal protein levels. C57BL/6J mice were tested in the BM and in the MTM, hippocampi were extirpated 6 h following the probe trials each and proteins were extracted for gel-based proteomic analysis. Mice learned the task in both paradigms. When hippocampal protein levels were compared between mazes, proteins from several pathways, including signalling, chaperone, metabolic and other cascades, significant differences were observed between protein levels in the two spatial memory tasks. The use of yoked controls revealed that the proteins were linked to spatial memory. It is intriguing to observe that hippocampal protein levels in the mouse tested for spatial memory are task dependent.

Proteomics and parasite–host interactions

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With the great progress of genomic sequencing projects during last few years, large datasets of genomic information for several parasites are now available. These genomic databases give investigators the extraordinary ability to analyze parasite proteins and parasite-host interactions by proteomic method, which is a powerful new approach that developed by the advances in protein separation/large-scale protein expression, mass spectrometry, protein microarray and computer-based search algorithms.

Food-born parasites including *Toxoplasma gondii, Cryptosporidium parvum, Clonorchis sinensis, Angiostrongylus cantontensis* cause substantial or subclinical diseases in human. These food-born parasites all discharge secretory proteins to mediate parasite-host interactions and pathogenic infections. Thus, a better understanding of these parasite-host interactions could lead to the development of novel preventions/treatments for parasite diseases. However, to date the detailed information and molecular mechanism of parasite-host interactions remain poorly understood. Aiming at these problems, and trying to identify novel target-proteins for the prevention/treatment of parasite diseases, we carry out a government-funded project (Chinese National key Basic Research Project, 973) of investigating the parasite-host interactions medicated by the secretory proteins from food-born parasites of *Toxoplasma gondii, Cryptosporidium parvum, Clonorchis sinensis, Angiostrongylus cantontensis* through cutting-edge proteomic technologies.

Solid state isotope exchange hydrogen for deuterium and tritium in peptides and proteins

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The investigation of the high temperature solid-state catalytic isotopic exchange reaction (HSCIE) under the action of spillover hydrogen is presented. This isotopic exchange in peptides and proteins can to be conducted without racemization. The HSCIE reaction is used both for the production of highly tritium labeled proteins (hemoglobin, insulin, interferon) and for studying the three-dimensional interaction of their subunits. The HSCIE reaction allows the areas of contact to be revealed in protein complexes. HSCIE was used to produce evenly tritium labeled biologically active peptides of 50–150 Ci/mmol molar radioactivities. Tritium label distribution over all amino acids in these peptides allows practically all the possible products of their fermentative hydrolysis to be simultaneously determined. The suggested method based on the employment of evenly labeled peptides makes it possible both to count the concentration of peptides in tissues in vivo and in vitro and to evaluate the activity of the enzymes participating in their degradation. Data on the pharmacokinetics of neuroactive peptides in brain tissues for different in vivo introduction modes are discussed. Evenly tritium labeled peptides are used in radioligand analysis. They are used in screening conducted for the search of the most perspective medicinal drugs of peptide nature. The HSCIE reaction is used for the production of a wide variety of tritium labeled selective ligands for glutamine, serotonine, nicotine and dophamine receptors. The preparations have high specific radioactivity and retain completely their physiological activity.

Characterisation of an aldo-keto-reductase that forms the subunit of the shaker potassium channel

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The shaker potassium channels are involved in various cellular signalling processes and have been implicated in disease states. The beta-subunit of the shaker potassium channel (Kvβ2) shows a high level of sequence homology with the aldo-keto reductase group of proteins. Recent studies have shown this protein to be catalytically active in aldehyde reduction and that channel function is modulated when Kvβ2-bound NADPH is oxidized. The present study has explored the substrate specificity of this subunit using a fluorimetric assay. It was found to possess broad specificity for the reduction of aromatic aldehyde substrates such as 2, 3 and 4-nitrobenzaldehydes, 4-cyanobenzaldehyde, 4-hydroxybenzaldehyde, pyridine 2 aldehyde and benzaldehyde. The presence of an electron withdrawing group at the position para to the aldehyde in aromatic compounds facilitated reduction. Aliphatic aldehydes were found to be poor substrates compared to the aromatic ones with 2-methylbutyraldehyde being the best. No clear evidence of an ability to dismutate aldehydes was found. The formation of reduced product (4-nitrobenzyl alcohol) by Kvβ2 was shown using 4-nitrobenzaldehyde as substrate. The activity of the enzyme was not inhibited by sodium valproate at a concentration of 250 μM.

Label-free quantitation with 2DB

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Proteomics aims to elucidate the complement to a genome including spatial, temporal, and other protein expression patterns. In this context, the differential expression of proteins under different physiological conditions, presents key information. Today, protein expression is generally investigated using mass spectrometry. Differential protein expression is investigated using either a differential labelling approach such as stable isotope labelling or a label-free setup such as spectral counts.

2DB is a database to store, analyze, and present data from mass spectrometric experiments. Spectral counting has been part of 2DB but more targeted analysis can now be performed with a new quantification tool presented here. General information about protein expression profiles within an experiment can be investigated in order to determine proteolytic processing and proteotypic peptides among other possibilities. Differential protein expression can be investigated using a new label-free approach amending spectral counting with abundance information in form of the summed total ion current. Furthermore, fractions in different experiments can be freely combined for analysis. The number of experiments is also not limited which allows for time series experiments. The software, depending on an installation of 2DB, is now part of the installation and as such freely available.

Establishment of platform for Cbl-b screening

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Systemic lupus erythematosus (SLE) is one of systemic autoimmune diseases. The aetiopathogenesis is unclear. The syndrome is influenced by genetic factors, sex hormones and environmental factors. There are several biochemical abnormalities in SLE patients. The gene transcription program is trigger an altered in T cells isolated from patients. That results in favoring functions of activation and effector.

C-Cbl, Cbl-b and Cbl-3 (Cbl-c) distributed in mammalian are casitas B-lineage lymphoma (Cbl) protein family. There is highly conserve in N terminal regions, including tyrosine kinase binding domain, linker and ring finger. Additionally, Cbl-b could be an E3 ligase and plays a negative regulatory role of T cell activation by controling the threshold. On SLE patients, the c2672 g mutation of *cbl-b* RNA was found and truncated *cbl-b* RNA was predicted. In the report, the exon/intron junction of *cbl-b* will be examined and the RACE will be performed for identifying the RNA truncation.

How to assemble a protein tag for purification, crystallisation and phasing

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During the last decade, several affinity protein tags consisting of short peptides or whole domains have been developed, which are included as an additive protein sequence on the C- or/and N-terminus. Their highly specific strong affinity affords a one-step purification with minimal effect on biological activity. On the other hand, protein tags are often disordered in the crystal structure and complicate protein crystallisation.

Experimental phasing of macromolecules by anomalous dispersion requires well ordered atoms in the crystal lattice. A preorganized protein tag with metal chelating properties can bind anomalous scatterers in a determinate way and enhance SAD and MAD data quality using synchrotron radiation. As a welcome side-effect such protein tags could adopt stable conformation in crystals and help the crystallization process. Therefore, the tags have to be ordered in the crystal structure.

Here we report polypeptides synthesized by solid phase peptide synthesis which may prove useful for both metal affinity chromatography and macromolecular phasing as well. A distinct secondary structure in terms of a-helices and β-hairpins could be confirmed by circular dichroism spectroscopy. Their chelating properties could be proved by Ni, Zn and Cu affinity columns.

For future investigation we plan to use proteins fused with our polypeptides to check IMAC behavior under real conditions, to validate crystal growth promotion and to get SAD and MAD data.

Analysis of hCGβcf glycosylation in normal and aberrant pregnancy by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Background: Metabolism of human chorionic gonadotropin (hCG) results in the urinary excretion of hCG β-core fragment (hCGβcf). hCGβcf retains many antigenic structures from the original β-subunit of hCG (hCGβ) as well as two metabolically processed N-linked oligosaccharides. Hyperglycosylation of hCGβ has been associated with aberrant pregnancy, especially malignant disease.
Methods: We have previously characterized the carbohydrate content of hCGβcf from pooled pregnancy urine using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS). Here, a similar methodology was used to compare the carbohydrate content of hCGβcf isolated from individuals with different pregnancy conditions: urinary hCGβcf from molar, hyperemesis gravidarum, and normal pregnancy was isolated by ion exchange chromatography, reduced with dithiothreitol, and subjected to MALDI TOF MS.
Results: Reduction of the disulfide linkages of hCGβcf resulted in the separation of the non-glycosylated β55-92 chain from the glycosylated β6-40 chain. The mass spectra revealed a set of resolved mass structures between 4156.8 and 5840.6 Da. The peak at 4156.8 Da corresponded to the non-glycosylated peptide of hCGβcf, β55-92, while the remaining structures represented the multiple glycosylated forms of the peptide β6-40. Prediction of glycoform structures was achieved by subtraction of the corresponding mass of the primary amino sequence of β6-40 from the observed m/z values corresponding to the glycosylated isoforms. Each sample contained 8-11 glycosylated forms of the β6-40 peptide.
Conclusions: All samples contained common and unique glycostructures. However, hCGβcf glycoforms possessing triantennary oligosaccharides were more abundant in aberrant pregnancy samples, reaching 16.5% in aberrant versus 1.6% in normal pregnancy.

Long-lived protein chloramines: transhalogenation reactions

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In activated neutrophils, myeloperoxidase catalyzes the reaction of H_2O_2 with Cl to produce hypochlorous acid (HOCl)—the bacteria killing agent. Reactive HOCl reacts reversible with the protein free lysine moieties to give *N*-monochloro- and dichloramines. *N*-chloramine formation could be reversed by reaction with thiols (cysteine, glutathione) and sulfides (methionine). In this process chloramines is reduced back to amine moieties. Chlorination however, promotes dose dependent covalent or non-covalent aggregation of proteins. The modified monochlorodimedone (MCD) method was applied in order to estimate the concentration of BSA mono- and dichloramines and to monitor the transhalogenation reaction from BSA chloramines to taurine, NH_4^+ and to cytochrome C. It was established (MCD method) that BSA monochloramines are the main products of BSA chlorination (pH = 5.4 and 7.4, 0.1–0.6 mM HOCl) and that the chlorination provide the mixture of monomeric BSA chloramines (R_H = 3.8 nm) and higher molecular aggregates (R_H = 6–9 nm) as was shown with use of the dynamic laser light scattering method (DLS). It was also shown that methionine does not dissociate BSA aggregates whereas reduced glutathione facilitates the dissociation process (DLS).

Our results confirm that protein chloramines are relatively stable (hours) and proved that chlorine atoms from *N*-chloramine BSA moieties are efficiently transferred to the low molecular amines (taurine) or ammonium salts with concomitant taurine and ammonium chloramines formation (10–20%). Also the ammonium chloramines (NH_2Cl) formation was detected/monitored with indophenol method. Results suggest that in phagocyte vacuole (pH = 5.3) the efficient transhalogenations reactions could produce toxic cell-penetrating low-molecular chloramines.

Identifying substrate specificity of a protein methyltransferase using a reverse chemical-genetics approach

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Methylation is a key post-translational modification of lysine and arginine residues in histone amino termini that regulates the organization of chromatin structure and gene activity in a wide variety of diseases. The aim of our project is to use a reverse chemical-genetics approach in order to identify the substrate specificity of histone methyltransferases through synthesis of enlarged analogues of the cofactor *S*-adenosyl-L-methionine (SAM) designed to fit complementary modified forms of protein methylases. The main focus of our research has been SET7/9, a lysine *mono*-methyltransferase which catalyzes the transfer of a methyl group to the unmodified histone H3-K4 lysine residue as well as residues in the transcription factor p53 and TAF10.

Site-directed mutagenesis has carried out to introduce mutations in SET7/9 that accommodate the enlarged analogues of SAM specifically. Full-length, HIS-tagged wildtype and mutant SET7/9 were expressed in *E. coli* and purified to optimised yields. Their catalytic activity was compared qualitatively or quantitatively using a fluorescent methyltransferase assay. Data on the binding affinity of natural SAM and a synthetic H3 peptide to wildtype and mutant forms of SET7/9 will be presented. A series of 5'- aziridino adenosines, analogues of SAM have been chemically synthesised as precursors which spontaneously convert to the active compound in situ. The effect of the analogues on the catalytic activity of wildtype SET7/9 has been assayed in-vitro using quantitative fluorescent assays and the interaction between the analogues and the H3 peptide substrate has been analysed by MALDI mass spectrometry.

Synthesis

Helical structures of *N*-alkylated poly(*p*-benzamide)s

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Poly(*p*-benzamide)s **1** bearing an *N*-(*S*)-2-(methoxyethoxyethoxy)propyl group as a chiral side chain were synthesized by chain-growth polycondensation methodology. The polyamides exhibited well-defined molecular weights with narrow polydispersities. In the solid states, the results of X-ray crystallographic analysis of 4-(methylamino)benzoic acid oligomers substantiated that they have a helical conformation with three monomer units per turn. Solutions of the polyamides in several organic solvents (CH_3CN, $CHCl_3$, and CH_3OH) showed dispersion type CD signals characteristic of coupled-oscillator and much larger as compared with the corresponding monomer. The CD signals (plus at around 300 nm and minus at ca. 260 nm) were dependent on the temperature and molecular weight of the polyamides but independent of the solvent, as far as examined; signals were more intense the larger the molecular weight and weaker the higher the temperature. An exciton model analysis of the absorption and CD spectra provided a clear-cut picture for the secondary structure of these polyamides in solution that the *N*-alkylated poly(*p*-benzamide)s possess a right-handed helical conformation ((*P*)-helix) with three residues per turn.

The new method for diastereoselective synthesis of pyrazolidines with N- and C-amino acids substitutents

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Pyrazolidine derivatives with an amino acid substituent at the ring nitrogen atom are very promising as fibrinogen receptors antagonists but the method for their synthesis from amino acid hydrazides is extremely limited.

We have found that *N*-phenyl-*N*-amino acid's pyrazolidines **1** may be obtained from 1 *N*-acetyl-5-(2-hydroxy-2-arylethyl)-3-methyl-2 *N*-phenylpyrazolidines **2a,b** or regioisomeric 1 *N*-acetyl-3-(2-hydroxy-2-arylethyl)-5-methyl-2 *N*-phenylpyrazolidines **3a,b**, which are readily available from racemic regioisomeric 1 *N*-acetyl-3-(2-oxo-2-arylethyl)-5-methyl-2 *N*-phenylpyrazolidines **4a** and 1 *N*-acetyl-5-(2-oxo-2-arylethyl)-3-methyl-2 *N*-phenylpyrazolidines methylpyrazolidinketones **4b**. Really, the conditions of diastereoselective reduction of the methylpyrazolidinketones **4** are founded. The reduction of the ketones **4** by sodium borohydride on the surface of an adsorbent [Al_2O_3] leads to the advantage of the one type of *trans*-diastereomers of hydroxy-phenylpyrazolidines **2a, 3a**. Alternatively the reduction of the ketones **4** by lithium hydride-tri-*tert*-butoxyaluminate in solution leads to the advantage of another one—diastereomers **2b, 3b**. The action of lithium aluminum hydride under mild conditions was used to remove the *N*-acetyl protective group and free *N*-phenylpyrazolidines **5** were prepared. The resultant pyrazolidines **5** converts to give stable *N*-phenylisothiocarbamoylpyrazolidines derivatives in high yield.

We were able also to carry out the reaction of unstable free *N*-phenylpyrazolidines **4** with *N*-Cbz-a.a. [Gly, (*S*)-Ala, (*S*)-Phe, (*S*)-Try)] under mild conditions only by using the Mukaiyama method, which permits the preparation of a set of different amino acids derivatives **1** (f. ex. 1-((*S*)-*N*-Benzyloxycarbonylalanyl)-5-(2-hydroxy-2-phenylethyl)-3-methyl-2-phenylpyrazolidine for (*S*)-Ala). All other reported methods for the acylation of amino acids did not give positive results.

From other side diastereomerically pure C-amino acid's pyrazolidines **6** after direct reductive amination of pyrazolidine alkylketones **1** with free amino acids were prepared [(1′*S*,2*S*,3*S*,5*S*)-1-acetyl-3-(2-(1′-benzylcarboxymethylamino)-propyl)-5-methyl-2-phenylpyrazolidine for (*S*)-Phe]. An X-ray crystallographic analysis was carried out for some of compounds and theirs absolute configurations were determined. All new compounds are potentially physiologically active.

The work was carried out with the financial support of the Branch of Chemistry and Material Science of the Russian Academy of Sciences (program No. 10, project "Directed Synthesis of Chiral Biogenic Amines Containing an Azolidine Ring").

Synthesis of the new bicyclic Pro-Glu chimeras by consecutive ester enolate alkylation

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New method for the synthesis of the conformationally restricted Pro-Glu chimeras has been developed. The target compounds possessing 7-azabicyclo[2.2.1]heptane and 8-azabicyclo[3.2.1]octane skeletons have been synthesized using a strategy of the consecutive stepwise bis-alkylation of the correspondingly protected pyrrolidine-2,5-dicarboxylate. While 1-bromo-2-chloroethane and 3-chloro-2-(chloromethyl)prop-1-ene were found to be useful to form the target cyclic compounds, 1,3-diiodopropane, 1-bromo-3-chloropropane as well as diiodomethane and bromochloromethane failed to give the corresponding bicyclic products. Influence of the N-protected group on the efficiency of the cyclization process has been investigated.

Stereospecific and flexible asymmetric synthesis of *cis*- and *trans*-3-hydroxy-pipecolic acids and analogs

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Both *cis*- and *trans*-3-hydroxy-L-pipecolic acids are synthesized from a common chiral intermediate **7** via a short and flexible route.

Stereospecific inversion of 3-OH is achieved by the formation of an oxazoline followed by acidic ring cleavage. The overall yields are 27% and 30%, respectively, in 10 and 12 linear steps. Several versatile chiral building blocks are also accessible via this diastereodivergent synthesis. Unlike chiral pool approach, our synthetic strategy is not limited by the availability of starting materials.

Synthesis of alkaloids from amino acids, using a one pot decarboxylation-alkylation reaction as the key step

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Amino Acids are useful synthetic intermediates. By using a sequential process which couples a decarboxylation reaction with the introduction of alkyl chains, commercial amino acids can be readily transformed into alkaloid precursors. An asymmetric version of this process was developed to prepare optically pure cytotoxic alkaloids and other analogues of bioactive alkaloids.

Transports

Glutamate regulates the monocarboxylate transporter (MCT4) protein levels in Bergmann glia

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L-Glutamate, the main excitatory neurotransmitter in the vertebrate brain exerts its action through specific membrane receptors and transporters that are expressed both in neurons and glia cells. The brain metabolic responses to activity are largely dependent on astrocytes through a coupling mechanism between neuronal activity and glial activation of aerobic glycolisis with a subsequent lactate release. The neuronal lactate consumption completes the cycle nowadays known as the astrocyte-neuron lactate shuttle. In order to gain insight into this coupling mechanism and its regulation by the major excitatory transmitter, using the well-characterized model of chick cerebellar Bergmann glia cultures we analyzed the protein levels of the monocarboxylate transporter 4 (MCT4). A time and dose-dependent up-regulation of the transporter protein levels was found. Noticeably, the effect reaches its maximal value after 30 min of Glutamate treatment, suggesting a post-transcriptional effect. Pharmacological experiments demonstrate the involvement of ionotropic and metabotropic receptors as well as the participation of the Na-dependent glutamate transporter (GLAST/EAAT1) in the Glutamate effect. The present results strengthen the notion of a critical involvement of glia cells in brain glucose metabolism.

Altered striatal glutamate transporter functioning could explain aberrant glutamatergic neurotransmission in striatum of hemi-Parkinson rat model

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Important mediators in the pathogenesis of Parkinson's disease are oxidative stress and excitotoxicity. Increased glutamate concentrations can be linked to both processes. Extracellular glutamate concentrations are mainly determined by an interplay between vesicular glutamate transporters (VGLUTs), glial high-affinity Na^+/K^+-dependent glutamate transporters (GLAST and GLT-1) and the cystine/glutamate antiporter. Using semi-quantitative Western blotting, we studied the expression levels of VGLUT1, VGLUT2, GLAST and GLT-1 in the striatum of rats at different survival times (3, 5 and 12 weeks) after unilateral 6-OHDA injection into the medial forebrain bundle (hemi-Parkinson rat model). The significant bilateral increase in GLT-1 expression that we observed at 3 and 12 weeks post-lesion, was translated into an increased reuptake activity, as revealed by in vivo microdialysis studies as well as in vitro reuptake studies in striatal synaptosomes. To determine the origin of the increased striatal expression level of VGLUT1 protein at 3 weeks post-lesion and decreased expression at 12 weeks, in situ hybridization experiments were performed to visualize VGLUT1 mRNA expression levels throughout the brain of hemi-Parkinson rats.

Our data suggest that the earlier observations by Meshul et al. (1999) of a biphasic change in extracellular striatal glutamate levels after 6-OHDA injection into the medial forebrain bundle, with increases 1 month post-lesion and decreases 3 months post-lesion, might be explained by an interplay of changes in the expression/functioning of all glutamate transporter families. Modulation of one or more of these transporters might help to normalize the extracellular glutamate levels and prevent further excitotoxic and oxidative damage.

vGLUT2 heterozygous mice show more susceptibility to clonic seizures induced by pentylenetetrazol

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Glutamate, the most abundant excitatory neurotransmitter in the central nervous system, is well known to be implicated in epileptic seizures. Therefore, impairments in glutamate transport could have an involvement in the mechanism of epileptogenesis. The uptake of glutamate into synaptic vesicles is mediated by vesicular glutamate transporters (vGLUTs). There are three known vGLUT isoforms, vGLUT1-3. In this study, we are particularly interested in the vGLUT2 isoform. We investigated the possible role of vGLUT2 in pentylenetetrazol (PTZ)-induced seizure generation. Seizure threshold of PTZ was compared in vGLUT2 heterozygous knock out (HET) and wild type (WT) mice. In comparison with their WT littermates a lower dose of PTZ was needed in the vGLUT2 HET mice until the onset of the first myoclonic jerk. The threshold for PTZ-induced clonic seizure activity was also lower in the vGLUT2 HET mice. These results indicate, for the first time, that vGLUT2 is likely involved in the epileptogenesis of generalized seizures.